Modulation of dendritic cell function by the fungal quorum sensing molecule farnesol

Dissertation

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by Wolfgang Vivas

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Dissertation examiners

1. Prof. Dr. Oliver Kurzai, Julius-Maximilians University, Würzburg and Leibniz Institute for Natural Product Research and Infection Biology - Hans-Knöll Institute, Jena, Germany.
2. Prof. Dr. Marc Thilo Figge, Leibniz Institute for Natural Product Research and Infection Biology - Hans-Knöll Institute, Jena, Germany.
3. Prof. Dr. Doris Wilflingseder, Medical University of Innsbruck, Austria.

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1 Summary

Microorganisms communicate with each other through the secretion of molecules that accumulate in the environment and mediate specific responses upon reaching a specific threshold. This process has been termed quorum sensing and is essential to coordinate changes in behavior dependent on cell population density. Farnesol (FOH) is the first quorum sensing molecule described in a eukaryotic organism, and it is produced by the opportunistic pathogenic fungus *Candida albicans.* FOH regulates morphogenesis in *C. albicans*; however, we and others have described a new role of FOH as a compound with immunomodulatory properties. In particular, FOH showed to impair DC differentiation from monocytes, resulting in altered expression of surface markers and secretion of cytokines, thereby suppressing DC ability to induce T cell proliferation. However, the molecular mechanisms by which FOH modulates DC differentiation and maturation are poorly understood. The results of this study revealed that FOH regulates DC function through several signaling pathways. FOH modulates the expression of costimulatory molecules partially through activation of the nuclear receptors PPARγ, RARα and LXRα. In particular, FOH increased the expression of the lipid antigen-presenting molecule CD1d through activation of p38 MAPK and PPARγ/RARα signaling pathway. However, CD1d upregulation did not confer these cells an elevated capacity to activate invariant Natural Killer T (iNKT) cells. FOH-differentiated DC showed diminished secretion of IL-12 and increased IL-10 release. Interestingly, reconstitution of the IL-12/IL-10 cytokine milieu restored FOH-differentiated DC to activate iNKT, Th1 and regulatory T cells. Mechanistically, FOH modulates IL-10 and pro-inflammatory cytokines through activation of RARα, LXRα, MAPK and NF-κB signaling pathways. Altogether, our results showed that FOH induces paralysis of DC function through activation of nuclear receptors, MAPK and NF-κB signaling pathways. Since these cells play an important role in regulating the immune response during infection, this work supports the role of FOH as a virulence factor produced by *C. albicans* to overcome immune surveillance by DC.

2 Zussammenfassung

Mikroorganismen kommunizieren miteinander durch die Sekretion von Molekülen, die sich in der Umwelt ansammeln und bei Erreichen eines bestimmten Schwellwertes bestimmte Reaktionen vermitteln. Dieser Prozess wird als Quorum Sensing bezeichnet und dient dazu, Verhaltensänderungen in Abhängigkeit der Zellpopulationsdichte zu koordinieren. Farnesol (FOH) ist das erste Quorum-Sensing-Molekül, das in einem eukaryotischen Organismus beschrieben wurde. Es wird von dem opportunistischen und pathogenen Pilz *Candida albicans* produziert und dient der Regulation der Morphogenese. Wir und andere haben eine neue Funktion von FOH beschrieben, in der es als Verbindung mit immunmodulatorischen Eigenschaften dient. FOH beeinträchtigte die Differenzierung von Monozyten zu Dendritischen Zellen (DZ), was zu einer veränderten Expression von Oberflächenmarkern und zur Sekretion von Zytokinen führte, wodurch die Fähigkeit der DZ zur Induktion der T-Zell-Proliferation unterdrückt wurde. Die molekularen Mechanismen, über die FOH die Differenzierung und Reifung von DZ moduliert sind jedoch kaum bekannt. Die Ergebnisse dieser Studie zeigten, dass FOH die Funktion von DZ über mehrere Signalwege reguliert. FOH moduliert die Expression kostimulatorischer Moleküle teilweise durch Aktivierung der Nuklearrezeptoren PPARγ, RARα und LXRα. FOH erhöhte insbesondere die Expression des Lipidantigen-präsentierenden Moleküls CD1d durch Aktivierung des p38-MAPK- und PPARγ/RARα-Signalweges. Die Hochregulation von CD1d verlieh diesen Zellen jedoch keine erhöhte Fähigkeit, invariante natürliche Killer T (iNKT)- Zellen zu aktivieren. FOH-differenzierte DZ zeigten eine verminderte IL-12 und eine erhöhte IL-10 Sekretion. Interessanterweise befähigte die Rekonstitution des IL-12/IL-10-Zytokinmilieus die FOH-differenzierten DZ wieder, iNKT-, Th1- und regulatorische T-Zellen zu aktivieren. Mechanistisch modulierte FOH die Freisetzung von IL-10 und entzündungsfördernden Zytokinen durch Aktivierung von RARα-, LXRα-, MAPK- und NF-κB-Signalwegen. Insgesamt zeigten unsere Ergebnisse, dass FOH die Paralyse der DZ durch Aktivierung der Nuklearrezeptoren und über MAPK- und NF-κB -Signalwege induzierte. Da diese Zellen eine wichtige Rolle bei der Regulierung der Immunantwort bei Infektionen spielen, unterstützt diese Arbeit die Rolle von FOH als Virulenzfaktor, der von *C. albicans* produziert wird, um die Immunüberwachung durch DZ zu überwinden.

3 Introduction

3.1 *Candida albicans*

C. albicans is one of the most prominent fungal commensal and pathogen of humans. *C. albicans* colonizes the gastrointestinal tract, mouth, skin and the female reproductive tract (1, 2). To colonize these niches, the fungus is able to adapt to several environmental conditions, including oxygen levels, host microbiome or changes in pH (1). However, *C. albicans* can become pathogenic if the host develops specific risk factors, such as microbial dysbiosis, immunodeficiency, major surgery, the presence of venous catheter and antibiotics (2, 3). Indeed, *C. albicans* can cause a broad range of infections in human, including superficial infections, such as oral or vaginal candidiasis, and life-threatening systemic infections (1). *C. albicans* relies on several virulence factors, including the expression of adhesins and biofilm formation, secretion of cytolytic proteins and morphological plasticity (4-10). *C. albicans* is a polymorphic fungus that can grow as budding yeast, pseudophyphae and true-hyphae (1, 2). The yeast form is associated with colonization and dissemination, while the filamentous form is involved in adhesion, penetration and tissue invasion (5, 6). Different environmental conditions are known to influence morphological transition in *C. albicans*, such as pH, starvation, the presence of serum or N-acetylglucosamine, temperature and CO_2 levels (6). Moreover, morphogenesis is also modulated by quorum sensing (QS), a mechanism of microbial communication to coordinate population density-dependent changes in behavior. In QS, secreted molecules from growing microorganisms accumulate in the local environment and modulate specific responses once a critical threshold or concentration has been reached. The main QS molecules produced by *C. albicans* include tyrosol, farnesoic acid and farnesol (1, 11-13). Tyrosol decreases the lag phase growth and stimulates filamentation as well as biofilm formation. However, its effects are only observed in absence of farnesol (11, 14). Farnesoic acid and farnesol inhibits the switch from yeast to hyphae form in a growing population. However, the effect of farnesoic acid is less than farnesol and only produced by one strain of *C. albicans* (12).

3.2 Farnesol production by *Candida albicans*

Farnesol (FOH) is a QS molecule produced mostly by *C. albicans* and inhibits the yeast to hyphae transition, which is one of the most important virulence factor associated with this fungus (1, 15). FOH is produced by *C. albicans* as a side product of the sterol biosynthetic pathway, which is essential for the membrane integrity of the fungus (16). Indeed, FOH is an acyclic sesquiterpene (isoprenoid/ 1-hydroxy-3, 7, 11-trimethyl-2, 6, 10-dodacatrien) produced by dephosphorylation of farnesol pyrophosphate (FPP), a key intermediate of the mevalonate pathway (17). Inhibition of the sterol synthetic pathway downstream of FOH induces accumulation of FPP, resulting in elevated concentration of FOH compared to untreated cells (18). Furthermore, inhibition of 3-hydroxy-methyl-glutaryl-CoA reductase (HMGR), necessary for mevalonate synthesis, reduces FOH production in *C. albicans* (19). Of the four existing isomers, only *E-E* FOH showed QS activity (20). Interestingly, FOH production is not unique to *C. albicans*. A study testing the capability of eight *Candida* species to produce FOH showed that *C. dubliniensis* can produce this QS molecule, but in much less quantities compared to *C. albicans* (21).

Production of FOH by *C. albicans* is likely dependent on the coordinated activation of several genes. Particularly, *DPP3* gene is known to be important for FOH biosynthesis in *C. albicans*. Production of FOH is significantly reduced in a DPP3 deletion mutant (22). Furthermore, overexpression of *DPP3* increases the morphogenic-inhibitory effects of FOH (23). Furthermore, synthesis was enhanced in deletion mutants of the morphogenic repressor genes *NRG1* and *TUP1*, as well as in the associated hyphal-maintenance gene *EED1* (24-26).

3.2.1 Effects of farnesol on *Candida albicans*

The most prominent effect of FOH is its ability to influence *C. albicans* morphology, by blocking the transition from yeast to filamentous cell without altering the growth rate (27, 28). Additionally, FOH inhibits biofilm formation in *C. albicans*. Ramage *et al.* showed that the effect of FOH on biofilm formation depends on the concentration of the compound and the initial time of adherence (29). While low FOH concentration did not affect biofilm formation, incubation with 300 µM FOH reduced *C. albicans* adherence and increased dispersal during the first 30 min of treatment. Interestingly, biofilm formation was impaired when cells were

incubated with 100 µM FOH for 24 hours (30). These results indicate that FOH treatment might promote *C. albicans* dispersal to colonize new niches. In addition to morphogenesis and biofilm formation, FOH regulates the expression of genes involved in drug resistance, iron transport, cell wall maintenance, oxidative stress, heat shock, surface hydrophobicity and iron transport (30-32).

Several reports have shown that FOH impacts multiple signaling pathways in *C. albicans*. The Ras1-cAMP-PKA cascade is one of the first signaling pathways identified to be influenced by farnesol. Ras proteins are GTPases localized in the plasma membrane which plays an important role in filamentation (33). Piispanen *et al.* (34) showed that FOH cleave Ras1 from the plasma membrane and the resulting soluble Ras1 reduced the ability to activate the catalytic activity of the adenylyl cyclase Cyr1p, supporting the formation of yeast cells. One important event on Ras1 localization in the plasma membrane involves farnesylation of the protein, which facilitates Cyr1p binding to Ras1 (35, 36). Thus, FOH could also block Ras1 activity via inhibition of farnesylation (37). Interestingly, FOH alters several other signaling pathways, such as activation of the general stress mitogen-activated protein kinase (MAPK) Hog1 and the two components signaling via the Cek1-MAPK pathway and stabilization of transcription factors like Tup1/Nrg1, leading to repression of filamentation (15, 25, 38-40). Finally, FOH modulates the activation of Efg1 and Czf1 transcription factors activities resulting in the transition from white to opaque cells, with opaque cells being killed at lower FOH concentrations (15, 41).

3.2.2 Farnesol effects on other microorganisms

FOH does not only affect *C. albicans* itself but alters other microorganisms and cell types (15). FOH blocks conidation and germination of *Aspergillus niger, Penicillium expansum,* and *Fusarium graminearum* via deregulation of G-protein/cAMP signaling pathway and induction of apoptosis. Interestingly, the induction of cell death by FOH in *Fusarium graminearum, Saccharomyces cerevisiae, A. nidulans* and *A. flavus* is a process dependent on metacaspase activation and ROS production (42-47). However, a different study found that FOH alters the membrane integrity of microorganisms due to its amphiphilic characteristic (48). Indeed, FOH treatment showed to blocks actin cytoskeleton formation in *A. fumigatus* by misplacing the hyphal tip localization of two prenylated Rho family GTPases,

AfRho1 and AfRho2, while inhibiting the cell wall integrity signaling pathway (48). Similarly, FOH increases drugs susceptibility of *Escherichia coli*, and *Staphylococcus aureus* via disruption of the cell membrane, resulting in the reduce formation of biofilms (49, 50).

3.2.3 Farnesol effects on mammalian cells

Besides FOH effects on *C. albicans* and other microorganisms, FOH has been shown to interact with mammalian cells. In particular, FOH induces apoptosis in several types of cancer cell lines, such as lung carcinoma cells, human oral squamous carcinoma cells, prostate cancer cells and breast cancer cells highlighting its relevance as a possible antineoplastic drug (51-54). However, some studies have found that FOH treatment also impacts viability of non-malignant cells. Particularly, FOH induces apoptosis and necrosis in spermatozoa (55). While FOH treatment reduced murine macrophages viability *in vitro*, the pro-apoptotic effect of this molecule on immune cells only occur at high concentrations (56, 57).

Although FOH inhibits filamentation in *C. albicans*, existing data suggest that FOH is likely a virulence factor promoting disease progression in systemic infections (22, 58). Treatment of *C. albicans* with subinhibitory concentrations of FOH-inducing azoles enhances virulence in a systemic candidiasis murine model (58). Furthermore, administration of FOH in mice infected with *C. albicans* reduced IL-12 and IFN-γ concentration, while increased kidney fungal burden and mortality (22). In accordance, infection with *C. albicans* strains that do not produce FOH showed reduced mortality in mice (22, 58). Likewise, treatment of mice with pravastatin, a HMG-CoA reductase inhibitor, decreased FOH production and enhanced viability during *C. albicans* infection (59). Interestingly, FOH treatment showed to be protective against oral candidiasis in mice.

Further evidences support immunomodulatory properties of FOH. Specifically, FOH treatment stimulates the chemokinesis of murine macrophages *in vitro* and *in vivo*, and these effects were enhanced in the presence of yeast cell wall components and aromatic alcohols (60). Interestingly, FOH administration with zymosan resulted in modulation of RAW264.7 macrophages genes expression, by enhancing IL-6, Toll-like receptor 2 (TLR2) and dectin-1 expression (61). However, Leonhardt *et al.* showed that FOH alters neutrophils, monocytes and dendritic cells functionality (56). FOH treatment promotes activation of

neutrophils and monocytes. However, this did not correlate with elevated phagocytic activity. In contrast, FOH dramatically impaired the differentiation of monocytes into dendritic cells. Particularly, FOH modified the expression of costimulatory and antigen-presenting molecules. These changes in surface molecules were accompanied by enhanced anti-inflammatory IL-10 and diminished IL-12 release and correlated with reduced capacity to induce T cell proliferation (56). Due to the increasing evidences of FOH as an important virulence factor and the relevance of dendritic cells in the anti-*Candida* immunity, this thesis focused in elucidating the molecular mechanisms in which FOH treatment orchestrates dendritic cells functionality.

3.3 Role of dendritic cells in anti-*Candida* immune responses

The innate immune system is the first-line of defense against invading pathogens, including *C. albicans.* While neutrophils and monocytes present one of the most important cellular components in the defense against this fungus, subsequent recognition and activation by dendritic cells leads to specific T cell anti-*Candida* immunity, which is important for fungal clearance (62, 63).

3.3.1 Dendritic cells

The orchestration of an effective immune response depends on dendritic cells (DC), a class of cells derived from bone-marrow that can be found in virtually most peripheral tissues, blood and lymphoid organs (64). DC are able to process extra- and intracellular antigens and present these via Major Histocompatibility Complex (MHC) molecules to T cells (65). Thus, DC are the bridge between the innate and adaptive immunity. DC population comprised of different types of cells, each with specific functions. They can be classified in plasmocytoid DC, myeloid conventional DC 1 (cDC1), myeloid conventional DC 2 (cDC2), Langerhans cells and monocyte-derived DC (64). Plasmocytoid DC are mainly present in blood and are specialized to sense and respond to viral infection by a rapid secretion of type I and type III interferons (66). Myeloid cDC1 are present in blood and tissues. These cells have the capacity to present antigens via MHC class I to promote $CD8^+$ T cells and NK cells activation through IL-12 production (64, 67). Myeloid cDC2 are present in blood, tissues and lymphoid organs. These cells are equipped with a range of C-type lectins, Toll-like receptors

(TLR), Nucleotide-binding oligomerization domain (NOD)-like receptors and Retinoic acid-inducible gene-I (RIG)-like receptors that allow them to recognize several different types of Pathogen-associated molecular patterns (PAMP). In contrast to cDC1, these cells have the capacity to produce high amounts of IL-12, allowing them to activate Th1, Th17 and $CD8^+$ T cells (64). Langerhans cells are skin-resident cells, specialized in production of pro-inflammatory cytokines while also presenting antigens to $CD8^+$ T cells (64).

Several reports have shown that monocytes can migrate into tissues in response to local inflammation, rapidly acquiring key properties of DC such as expression of specific DC markers, present exogenous antigens to T cells and produce high amounts of inflammatory as well as anti-inflammatory cytokines (64). Monocytes differentiate into immature DC (iDC) specialized to engulf foreign antigens, resulting in antigens loaded into MHC molecules. Generation of mature DC (mDC) is achieved after activation of iDC with different antigens, mainly LPS, TNF-α or CD40L, leading to elevated expression of costimulatory molecules, migration to lymph nodes, antigen presentation to T cells and production of T cell-polarizing cytokines (68-70). Upon LPS recognition by TLR4, downstream adaptors are recruited into the intracellular domain of the receptor (Figure 1), such as the myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor protein (TRAM). Different TLR use different combination of adaptor proteins to determine downstream signaling. TLR 1, 2, 5 and 6 mainly use the MyD88-dependent pathway, while TLR4 use both downstream signaling pathway. MyD88 activation leads to recruitment and activation of the IRAK and TRAF6 kinases, which activate IκB kinase (IKK) and mitogen-activated protein kinase (MAPK) downstream signaling. IKK further phosphorylates ικB which leads to its degradation and subsequent nuclear translocation of the NF-κB transcription factor resulting in DC maturation by increasing the expression of costimulatory molecules and cytokines production (71-77). Activation of MAPK results in induction of the activator protein-1 (AP-1) transcription factor, which has also a role in the transcription of pro-inflammatory cytokines (71-73). Notably, MAPK regulates DC maturation through phosphorylation of NF-κB. Accordingly, inhibition of p38 and JNK MAPK activation blocked the upregulation of the costimulatory molecules and the release of pro-inflammatory cytokines induced by LPS stimulation, resulting in a reduced capacity to prime T cells (74, 78, 79). In contrast, the

MyD88-independent pathway leads to activation of a different transcription factor interferon response factor (IRF), which is mainly involved in the type-1 interferon production (71-73).

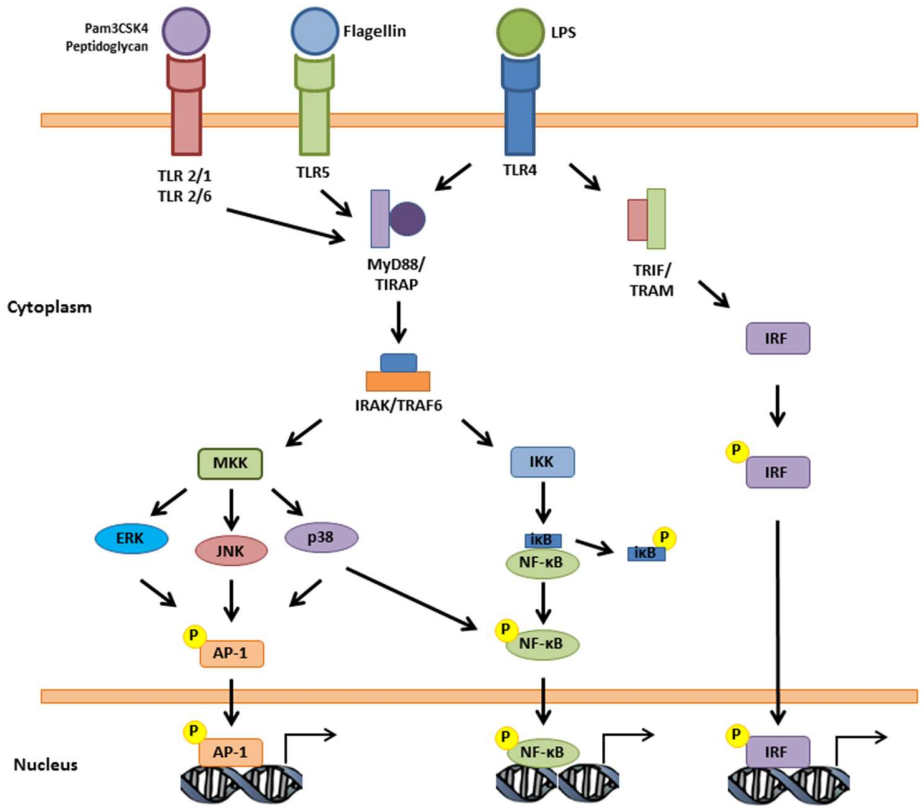

Figure 1. TLR signaling pathway

Maturation of DC is typically achieved through stimulation with TLR agonists. Upon recognition of TLR ligands, signaling is driven in a MyD88-dependent or independent manner. MyD88 activation leads to recruitment of IRAK and TRAF6, followed by activation of MAPK and IKK. IKK phosphorylates iκB, leading to degradation of iκB and nuclear translocation of NF-κB. The p38, JNK and ERK MAPK activate AP-1 and NF-κB transcription factors. The MyD88-independent pathway is mediated through the TRIF/TRAM adaptor protein complex, which enhances IRF nuclear translocation. Finally, AP-1, NF-κB and IRF mediate the transcription of genes involved in costimulatory molecules expression and cytokine production. Figure modified after (71-73)

Due to the relevance of monocytes-derived DC *in vivo*, the development of protocols for *in vitro* generation of these cells has been used successfully to study DC biology. $CD14^+$ monocytes can be isolated from donors and cultured in presence of GM-CSF and IL-4 for 6 days to obtain iDC. iDC generated by this method resemble the surface molecule expression and morphology observed *in vivo*. iDC are characterized by enhanced capacity to bind and

process antigens while expressing markers including group 1 CD1 (CD1a, CD1b and CD1c). Moreover, mDC retain expression of CD1 molecules and upregulate antigen-presenting and costimulatory molecules on their surface, including MHC class I and II, CD40, CD80, CD86 (64, 80-82).

3.3.2 DC antigen presentation and T cell polarization

DC have the capacity to efficiently present antigens to T cells. This process is in part dependent on the optimal expression of antigen-presenting molecules, including as MHC class I and II molecules. While MHC class I present peptide antigens to $CD8^+$ cytotoxic T cells, MHC class II activates $CD4^+$ T helper cells (67). MHC class II proteins include HLA-DR, HLA-DQ and HLA-DP, and are expressed on iDC and become highly up-regulated after iDC maturation. Efficient antigen presentation by DC correlates with the levels of these molecules on the surface (67, 83). Unlike MHC class I and II molecules which present peptides as antigens, members of the CD1 family present self- and foreign lipid antigens to T lymphocyte subsets. CD1 molecules are classified depended on their sequence homology into group 1 (CD1a-c), group 2 (CD1d) and group 3 (CD1e) (84, 85). While group 1 and 2 are expressed on the surface, group 3 is located intracellularly and might play a role in antigen-processing rather than presentation (84-86). Group 1 family members present lipid antigens to conventional T cells, while CD1d functions as antigen-presenting molecule to a specific T cell subset known as invariant Natural Killer T cells (iNKT) (87, 88).

In concert with signals triggered by peptide-MHC or lipid-CD1 complexes presentation, T cell activation requires a “second signal” triggered by costimulatory molecule interactions, mainly mediated by the expression of CD40, CD80 and CD86 on DC (89-91). Binding of CD80 and CD86 to their partner CD28 on T cells confers optimal production of IL-2, an important cytokine that promotes expansion and survival of T cells (90-92). However, the CD40/CD40L pathway also regulates T cell priming and differentiation. CD40 ligation on DC further increases the expression of costimulatory, adhesion and MHC molecules (89, 93). Consequently, DC produce an array of cytokines that direct $CD4^+$T cell differentiation into one of the different subsets of effector T cells, such as T helper 1 (Th1), Th2, Th17 or regulatory T cells (Treg) (94). Th1, Th2 and Th17 subsets are defined based on the different cytokine combinations that they secrete. Th1 cells development is induced when IL-12 is

present, and play an important role in activating monocytes, macrophages and $CD8^+$ T cells, via secretion of pro-inflammatory cytokines, including IFN-γ (95-97). In contrast, Th2 differentiation requires IL-4 and are involved in the recruitment of eosinophils, mast cells and basophils, while also promoting the activation of B cells (96). Th17 arise when the cytokine IL-6 and transforming growth factor (TGF-β) are present. Th17 cells secrete IL-17 and IL-22 and are known to be important in controlling fungal infections (98-100). Treg are crucial in the maintenance of peripheral tolerance, by the release of anti-inflammatory cytokines such as TGF-β and IL-10 (101). Treg cell population has been specially described through their expression of the transcription factor FOXP3, which is critical for the development and function of these cells (102, 103). DC promote tolerance by limiting effector T cells and promoting Treg differentiation in the periphery through various mechanisms including the production of immunosuppressive cytokines such as IL-10, IL-27 and TGF-β, and the expression of inhibitory molecules such as PD-L1, ILT2, ILT3 and ITL4 (104-107). In general terms, the interaction between PD-L1 and its receptor PD-1 inhibits immune cells activation via induction of apoptosis and T cell anergy (108). Moreover, engagement of the inhibitory receptors ILT2, ILT3 and ILT4 reduces the expression of costimulatory and antigen-presenting molecules, and inhibits the secretion of cytokines and chemokines in DC (109).

3.3.3 DC signaling and immunity against *Candida albicans*

DC play an important role in controlling mucocutaneous and systemic *C. albicans* infections (110-112). For *C. albicans,* dimorphism is a key trait involved in the interaction with DC. Ingestion of *C. albicans* yeast cells activates IL-12 production, promoting a priming of Th1 cells. In contrast, ingestion of hyphae leads to non-protective Th2 priming by suppressing IL-12 release and increasing IL-4 production (113). Moreover, immunization of mice with DC pulsed with *C. albicans* yeasts, but not hyphae, reduced the CFU observed in kidneys of *C. albicans* infected mice (113). While Th1 responses are known to be relevant during systemic infection, Th17 play an important role during mucocutaneous infection. Th17 cell activation by DC is induced by yeast cells in a process mainly dependent on the dectin-1/2-SYK-CARD9 signaling (99, 111, 114, 115).

DC effectively recognize *C. albicans* through the expression of a wide range of pattern recognition receptors (PRR), including the C-type lectin receptors (CLR) dectin-1, dectin-2 and cell-specific intracellular adhesion molecule-3-grabbing non integrin (DC-SIGN), as well as the mannose receptor (MR), Mincle, Galectin-3, TLR2, TLR4, TLR6, and complement receptor 3 (CR3). Altogether, these receptors efficiently recognize structural conserved moieties expressed on the cell wall of *C. albicans*, such as β-glucans, phospholipomannan, *N*-linked and *O*-linked mannans (116-122). However, the CLR dectin-1 and dectin-2 play an essential role in morphology-specific recognition of *C. albicans*. While dectin-1 recognizes β-glucan residues mainly expressed in yeast cells, dectin-2 recognizes mannose structures present mostly in hyphae forms (123). Although these receptors have certain differences in the downstream signaling compared to TLR, they also share common pathways including activation of NLRP inflammasomes, MAPK, NF-κB and AP-1 transcription factors, resulting in enhanced the expression of costimulatory molecules and secretion of cytokines (Figure 2) (123-126).

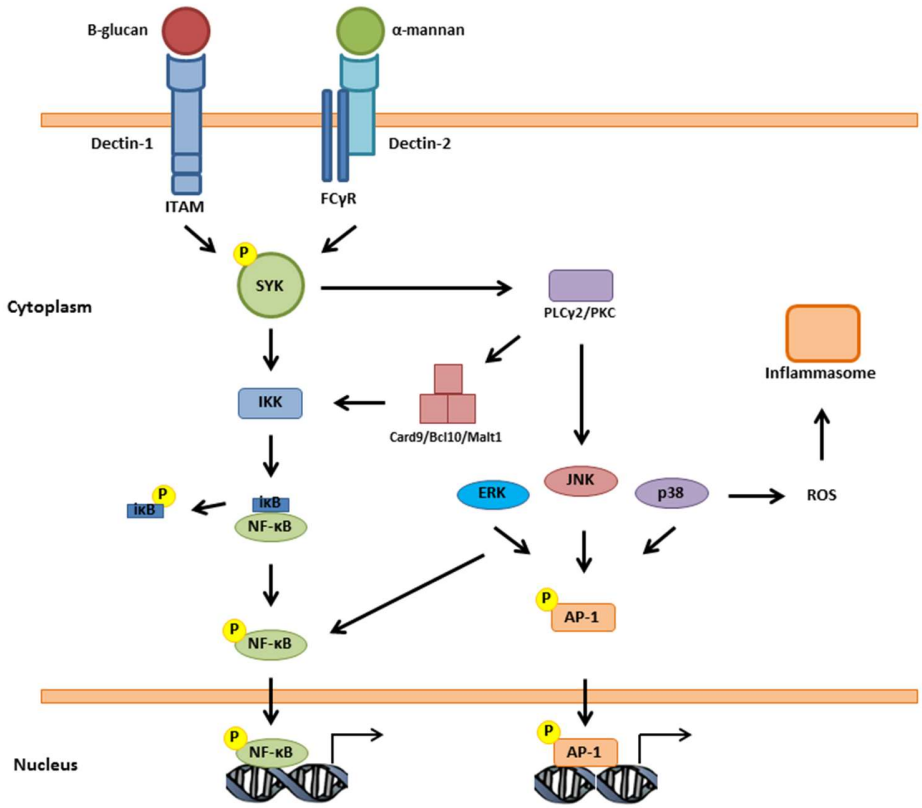

Figure 2. Dectin-1 and dectin-2 signaling pathway

Recognition of *C. albicans* antigens by dectin-1 and dectin-2 leads to phosphorylation of the spleen tyrosine kinase (Syk). Syk phosphorylation leads to NF-κB nuclear translocation by removing its inhibitor iκB through the IKK complex. Syk can also activate the PLCγ2 signaling leading to PKC activation that controls ROS/NLRP3 inflammasome, AP-1 activation via MAPK phosphorylation and activation of CARD9/Bcl10/Malt1 complex. The Card9 complex regulates NF-κB activity through the iKK complex. Resultant activation of NF-κB and AP-1 leads to expression of cytokines and chemokines. Figure modified after (126).

3.3.4 Nuclear receptors and their role in DC maturation and immune response against fungal infections

3.3.4.1 Nuclear receptors

Nuclear receptors (NR) are transcription factors activated by lipid-soluble ligands, which regulate the expression of several genes involved in diverse processes, including differentiation, development, proliferation, metabolism and inflammation (127-130). NR transcriptional activity is achieved by a conserved structure consisting of regions with specific functions as depicted in figure 3. All NR contain an activation function region 1 (AF-1), a hypervariable N-terminal region which serves as a target for post-translational modifications, such as phosphorylation and SUMOylation, facilitating ligand-independent activation function. Furthermore, NR contain a highly conserved DNA-binding domain (DBD) which allows the binding to specific DNA sequences known as hormone response element. NR ligands bind to the C-terminal region containing the ligand-binding domain (LBD), which also mediates dimerization with another NR. Finally, the AF-2 conserved region located near the C-terminus regulates ligand-dependent activation of NR, by controlling the binding of coactivator and corepressor complexes (127, 128, 130, 131).

Figure 3. Nuclear receptor structure

The superfamily of NR is subdivides based on their ligands as “classical” endocrine receptors, orphan receptors and adopted orphan receptors (127, 128, 132). Classical receptors include the retinoic acid receptor (RAR), vitamin D receptor (VDR), thyroid hormone receptors (TR), which are activated by *all-trans* retinoic acid, 1α25-dihydroxyvitamin D, and thyroid hormones, respectively (127). In contrast, the ligands for orphan receptors are not known. However, several NR became “adopted” following the description of several ligands, including peroxisome proliferator activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR) and retinoid X receptor (RXR), activated mainly by metabolites such as fatty acids, oxysterols and bile acids (127, 128, 132).

One-third of NR form an obligatory heterodimeric partner with RXR and can be further classified in two groups depending on the ligand binding properties: permissive and non-permissive NR. While permissive NR, including PPAR and LXR can be activated either by their own ligands or RXR ligands, non-permissive NR, such as RAR, VDR and TR become only activated by their own agonists (127, 128). RXR heterodimers are located in the nucleus and bind to specific nucleotide sequences, known as hormone response elements, in the absence of ligands inhibiting transcriptional activation by the recruitment of corepressor molecules (Figure 4A) (127). However, the presence of ligands promotes dissociation of the corepressors and recruitment of coactivator complexes necessary for optimal transcriptional activity (Figure 4B). Alternatively, some ligands can trigger corepressor molecules recruitment, which blocks transcriptional properties of NR (Figure 4C). Finally, other NR suppress activation of different transcription factors in a process known as transrepression (Figure 4D). Particularly, SUMOylation of PPARγ and LXR triggers their recruitment to promotors of inflammatory transcription factors, including NF-κB and activator protein 1 (AP-1) and physically modulating their activation (127).

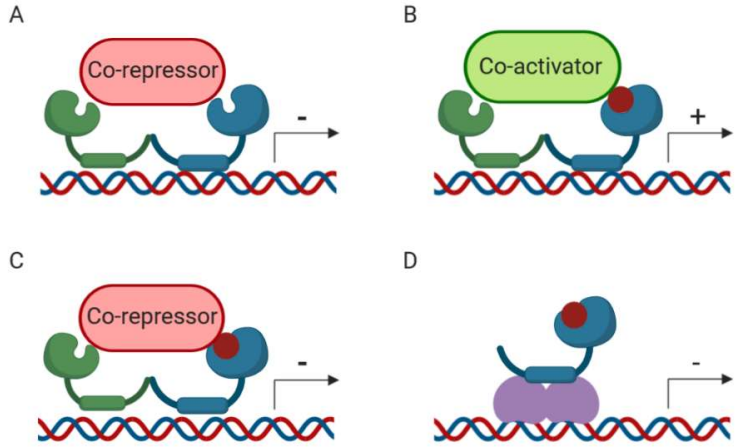

Figure 4. Nuclear receptor mechanism of action

Nuclear receptors and its partner, RXR, binds to specific DNA sequences and regulates the expression of several genes involved in multiple processes. Nuclear receptors activation is tightly regulated by the presence of its ligands. Liganded receptors can recruit co-repressors or co-activator complexes to enhance or inhibit gene expression. Alternatively, nuclear receptors might interfere with activation of other transcription factors in a process known as transrepression. Figure modified after (127).

3.3.4.1.1 Retinoid X receptor: RXR

Retinoic X receptor (RXR) is a unique member of NR because it forms heterodimers with several other NR, most of them requiring RXR as a mandatory partner for DNA binding and transcriptional activity. Thus, binding of ligands to permissive RXR heterodimers can potentially modify various pathways simultaneously, thereby modulating developmental, metabolic and immune processes (127, 133, 134). The most common ligand known is the vitamin A metabolite 9-cis retinoic acid (9-cis-RA) which is able to activate all three forms of RXR - RXRα, RXRβ and RXRγ (135). Nevertheless, several other synthetic and natural agonists have been described (136).

The regulation of immune function by this receptor was shown in human monocytes-derived DC (137). Particularly, activation of RXRα regulates the differentiation process from monocytes to DC by altering the expression of costimulatory and antigen-presenting molecules, reducing IL-12 release, and increasing IL-10 production in response to LPS stimulation (137, 138). Furthermore, activation of RXRα *in vivo* reduced DC migration to draining lymph nodes due to inhibition of CCR7 expression (139).

3.3.4.1.2 Retinoic acid receptor: RAR

Retinoic acid receptor (RAR) mediates all its functions by recognition of the active metabolite of vitamin A, *all-trans* retinoic acid (ATRA), or synthetic ligands which can activate the three isoforms – RARα, RARβ and RARγ. RAR plays a crucial role in the regulation of immune function, highlighted by higher rates of infections observed in vitamin A deficient patients (127, 140-143). RAR forms a non-permissive heterodimer with RXR that binds to the retinoic acid response element, consisting of two direct repeats of PuGGTCA spaced by five bases (144). Similar to other NR, RAR can interact with several other pathways which highlight its relevance in the regulation of multiple processes (145-147).

Several *in* vitro and *in vivo* studies suggested an anti-inflammatory effect of RAR activation on innate immune cells (148-150). RAR activation by ATRA or synthetic agonists promotes the differentiation of M2 macrophages expressing anti-inflammatory cytokines, while suppressing TNF-α and nitric oxide production (148). Moreover, activation of RAR by ATRA leads to a repression in IL-12 release via inhibition of NF-κB activation (149, 150).

RAR is also involved in modulating DC function. Particularly, DC derived from human cord blood and differentiated in presence of ATRA are characterized by a reduced IL-12 and increased IL-10 secretion (151). Furthermore, DC priming with ATRA enhances the release of TGF-β, which promotes expansion of Treg cells, highlighting the importance of ATRA as a metabolite with immune-suppressive potential (152, 153). Additionally, treatment of mouse splenic DC with ATRA promotes the expression of SOCS3, which attenuates the activation of p38 MAPK, and thereby, inhibits the release of IL-12, IL-6 and TNF-α (154). However, in presence of pro-inflammatory stimuli, ATRA treatment further increases the maturation of DC by activation of NF-κB (155). Interestingly, activation of RAR showed to coordinate several genes during the transition from monocytes to DC. Particularly, group 1 of CD1 genes (CD1a, CD1b and CD1c) was downmodulated, while CD1d showed upregulation upon ATRA treatment. Moreover, upregulation of CD1d correlated with an elevated capacity to activate invariant Natural Killer T cells (iNKT) (156). Finally, ATRA could be used as an adjuvant due to the enhanced DC migration to draining lymph node in a murine model (157).

Activation of RARα in immune cells modulates infection by pathogenic fungi. In particular, ATRA treatment diminished inflammation induced by monocytes infected with *C. albicans* by reducing the surface exposure of Dectin-1 and TLR2, together with a diminished

secretion of TNF-α, IL-6 and IL-12. The authors concluded that ATRA administration might be useful for prophylactic applications in septic patients infected with *C. albicans* (158).

3.3.4.1.3 Peroxisome proliferator activated receptors: PPAR

The family of peroxisome proliferator-activated receptors (PPAR) includes three isoforms: PPARα, PPARβ/δ and PPARγ (159). These isoforms differ among each other in their tissue expression. PPARα is involved in fatty acid metabolism processes, including β-oxidation pathway, fatty acid catabolism, lipogenesis and ketone body synthesis (160). Thereby, PPARα is expressed in metabolically active tissues, such as liver, heart, skeletal muscle, intestinal mucosa and brown adipose tissue (160, 161). Immune cells including monocytes, macrophages and lymphocytes also express this NR (162, 163). Although PPARβ/δ is ubiquitously expressed in all organs, it is highly present in the gastrointestinal tract (esophagus, liver, and intestines), kidneys and skeletal muscle (164). Similarly, PPARγ is expressed in white and brown adipose tissue, the large intestine, spleen, and immune cells, most prominently in macrophages and DC (127, 160, 165).

PPAR have a large cavity binding site, which is approximately 3-4 larger than other NR (166). Thereby, PPAR are able to bind several natural and synthetic ligands. In particular, PPARγ is activated by natural ligands such as unsaturated fatty acids, 15-hydroxy-eicosatetraenoic acid, 9- and 13-hydroxy-octadecadienoic acid, 15-deoxy Δ12,14-prostaglandin J2 and prostaglandin J2 (160, 165). Among synthetic ligands are the insulin sensitizers Thiazolidinediones (such as englitazone, ciglitazone, pioglitazonem troglitazone and rosiglitazone), non-steroidal anti-inflammatory drugs (i.e. indomethacin, fenoprofen, flufenamic acid) and other PPARγ modulators, so-called SPPARMs (Selective PPAR modulators) (165).

PPARγ is known to influence the development and functionality of monocytes and macrophages (167-171). Activation of PPARγ suppressed the production of pro-inflammatory cytokines, such as TNF, IL-6, IL-1β, IL-12, iNOS and MMP-9 in these cells (167-170). Interestingly, PPARγ also modulates macrophages migration in response to MCP-1, by suppressing CCR2 expression (171). PPARγ inhibits inflammation by physically interacting with other transcription factors involved in pro-inflammatory pathways, including NF-κB, AP-1, STAT-1 and NFAT, in a process known as transrepression. This process is

regulated by SUMOylation of PPARγ, which allows PPARγ to directly interact with the transcription factors, thereby preventing the binding to their response elements (172). Sequestration of coactivators, signaling through the IL-4 receptor, and altered activation of MAPK mediated by PPARγ seems to play an important role in suppressing production of inflammatory cytokines (127, 173-175).

Furthermore, PPARγ is well known to modulate DC functionality by altering the surface phenotype, cytokine secretion and T cell activation capacity. Particularly, treatment of DC with PPARγ agonists dampens the release of pro-inflammatory cytokines and chemokines, including TNF-α, IL-12, IL-5, IL-6, IP-10 (CXCL10) and RANTES (CCL5) (176, 177). Moreover, PPARγ activation enhances CD86 while downmodulates CD80 expression (87). However, the most important effect was observed in CD1 molecule expression pattern. While CD1a is downregulated, CD1d expression increases in PPARγ-instructed DC (87). Mechanistically, PPARγ modulates CD1d expression indirectly by enhancing the activity of aldehyde dehydrogenase (ALDH1A, RALDH) which is involved in ATRA production. ATRA binds to RAR and modulates the expression of CD1d (156). Furthermore, PPARγ activation increases the expression of cathepsin D, a lysosomal aspartyl protease, which improves lipid antigen processing and loading to the CD1d complex (178).

PPARγ may play either a beneficial or detrimental role during infection, mainly dependent on the pathogen involved (179-181). Majer *et al.* showed that administration of the synthetic PPARγ agonist pioglitazone improves viability of mice infected by *C. albicans* by suppressing neutrophils infiltration into the kidneys of infected mice (182). Furthermore, PPARγ activation increases the expression of dectin-1 and MR in macrophages. Accordingly, macrophages produce more ROS and have a higher capacity to uptake and kill *C. albicans* (183-185).

3.3.4.1.4 Liver X receptor: LXR

Liver X receptors (LXR) – namely LXRα and LXRβ - have a large hydrophobic cavity that enables the binding of cholesterol and several oxysterols. Thus, this NR play a key role in modulating metabolism and cholesterol homeostasis (186). LXR is highly expressed in the liver, intestine, adipose tissue, lung, the adrenal glands, kidney, lymphocytes, monocytes, macrophages and DC (127, 186). LXR forms a permissive heterodimer with RXR, and binds

to specific DNA sequences known as LXR-responsive elements (LXRE), consisting in direct repeats of AGTCA separated by 4 nucleotides (187).

Most of the studies focused on the role of LXR in modulating lipid metabolism. However, LXR activity also regulates immune responses, especially in macrophages and DC (188-192). LXR exerts anti-inflammatory effects in macrophages by inhibiting the expression of several pro-inflammatory molecules, such as IL-6, IL-1β, MCP-1, MCP-3, iNOS, and cyclooxygenase 2 in response to bacterial or LPS stimulation (193, 194). Similarly as PPARγ, LXRα can suppress inflammatory signaling by transrepression which requires SUMOylation of the receptor (195). Interestingly, these effects were only observed in short-term LXR stimulation. Long-term activation of LXR in human macrophages potentiates LPS responses, by increasing TLR4 expression, activation of MAPK and ROS production (196).

The role of LXR in DC biology is conflicting. Geyeregger *et al.* showed that treatment of monocytes with a synthetic LXR ligand impairs DC differentiation and maturation by reducing IL-12 and increasing IL-10 secretion (190). Moreover, these cells have a lower capacity to activate T-cell, in a process dependent on the reduction of the actin-bundling protein fascin (190). However, comprehensive transcriptional and functional analysis showed that differentiation of human DC in presence of LXR ligands promotes their capacity to activate $CD4^+$ T cells by increasing the expression of costimulatory molecules and pro-inflammatory cytokines production (IL-12, TNF-α, IL-6 and IL-8), in a process dependent on NF-κB activation (191). Interestingly, LXR has a crucial role in DC migration during inflammatory conditions due to the regulation of the ectoenzyme CD38 (192, 197).

Several reports showed that LXR play an important role in modulating the immune response against pathogenic bacteria, viruses and parasites (188, 198-202). However, only one report has addressed the role of LXR during infection by pathogenic fungi. Bobryshev *et al.* demonstrated that macrophages infected with *Chlamydia pneumoniae* showed reduction in LXRα expression, which correlated with higher formation of foam cells in atherosclerotic lesions (203).

In summary, NR modulate differentiation and maturation of DC. Furthermore, these receptors are involved in the interaction with pathogenic microorganisms. Nevertheless, only few reports have addressed the role of these transcription factors during fungal infections. Moreover, no study so far has evaluated the potential of microbial products produced by pathogenic fungi in modulating immune responses through NR activation.

4 Aims of the work

Farnesol, the first quorum sensing system described in eukaryotes, modulates the hyphae to yeast transition in the filamentous fungus *C. albicans*. However, farnesol also acts as a virulence factor during *C. albicans* infection, by altering immune cells function. Particularly, farnesol impairs dendritic cell differentiation and maturation process from monocytes. Nevertheless, the molecular mechanisms by which farnesol alters dendritic cell functionality remain elusive. In this work, the elucidation of signaling pathways activated by farnesol in dendritic cells was investigated.

The specific aims of this work were:

- Define molecular pathways modulated by farnesol through the available microarray data of dendritic cells differentiated in presence of farnesol
- Characterize the contribution of nuclear receptors to modulation of dendritic cell maturation by farnesol
- Analyze downstream inflammatory signaling pathways activated by farnesol during the maturation process of monocytes to dendritic cells
- Evaluate the impact of farnesol treatment on the ability of dendritic cells to activate different T cells subsets

5 Experimental procedures

5.1 Reagent preparation

Trans-trans, FOH was obtained as a 4M stock solution (Sigma-Aldrich) and then diluted in 100% methanol, followed by serial dilutions in RPMI + 5% FBS to the working concentrations of 50 µM and 100 µM. Concentrations of FOH were selected based on the observation that *C. albicans* can release up to 55 µM FOH in *in vitro* culture, and concentrations of 10-250 µM have been shown to modulate morphology transition in *C. albicans* (204, 205). The following antagonists for nuclear receptors were obtained from Tocris Bioscience: GW9662 (PPARγ antagonist, 10 µM), AGN193109 (RARα antagonist, 10 µM), GSK2033 (LXRα antagonist, 1 µM). Nuclear receptor agonists were: Rosiglitazone (PPARγ agonist, 5 µM, Enzo Life Sciences), AM580 (RARα agonist, 100 nM, Sigma-Aldrich) and GW3965 (LXRα agonist, 1 µM, Tocris Bioscience). Methanol was used as a solvent control indicated as mock-treated DC. Lipopolysaccharide (LPS, Sigma-Aldrich), peptidoglycan (Sigma-Aldrich), flagellin (Sigma-Aldrich), zymosan (InvivoGen) and Pam3CSK4 (R&D Systems) were diluted in water before application. α-galactosylceramide (α-GalCer) was obtained from AbCam and diluted in DMSO.

5.2 Dendritic cell generation and maturation

Human monocytes were isolated from buffy coats of healthy volunteers (provided by the Transfusion Medicine Department of the University Hospital Jena). First, Peripheral Blood Mononuclear Cells (PBMC) were obtained by density gradient centrifugation using BIOCOLL (Biochrom AG), and monocytes were positively selected by using anti-CD14-conjugated beads via a magnetic cell sorting system (MACS) (Miltenyi Biotech). Freshly isolated monocytes were resuspended into 6-well plates at a density of 2 x 10^6 cells per well and cultured in RPMI-1640 (Biochrom AG) with 10mM L-glutamine supplemented with 10% heat inactivated FBS (Biochrom AG), 800 U/ml GM-CSF (Leukine® Sargamostim) and 1000 U/ml IL-4 (Miltenyi Biotec). Cells were cultured for 6 days at 37°C and 5% CO_2 and half of media was changed at day 3. Ligands or solvent control were added to the cell culture starting from the first day. NR antagonists were added 1 hour prior FOH or solvent stimulation. iDC differentiation was confirmed by CD1a positive and CD14 negative staining.

To obtain matured dendritic cells (mDC), iDC were treated with 10 ng/ml LPS (Sigma-Aldrich) for 24 hours. To block NF-κB and MAPK activation, iDC were pre-incubated for 30 min prior LPS stimulation with the following inhibitors: SC75741 (NF-κB inhibitor, 5 µM, Cayman), SP600125 (JNK inhibitor, 10 µM, InvivoGen), SB203580 (p38 inhibitor, 20 µM, InvivoGen), and FR180204 (ERK1/2 inhibitor, 10 µM, Tocris). To evaluate Toll-like receptor and dectin-1 activation, iDC were stimulated for 24 hours with the following ligands: Pam3CSK4 (TLR 1/2 ligand, 10 µg/ml, R&D systems), peptidoglycan (TLR 2/6 ligand, 10 µg/ml, Sigma), flagellin (TLR 5 ligand, 100 ng/ml, Sigma) and zymosan (dectin-1 ligand, 100 µg/ml, InvivoGen) (Figure 5).

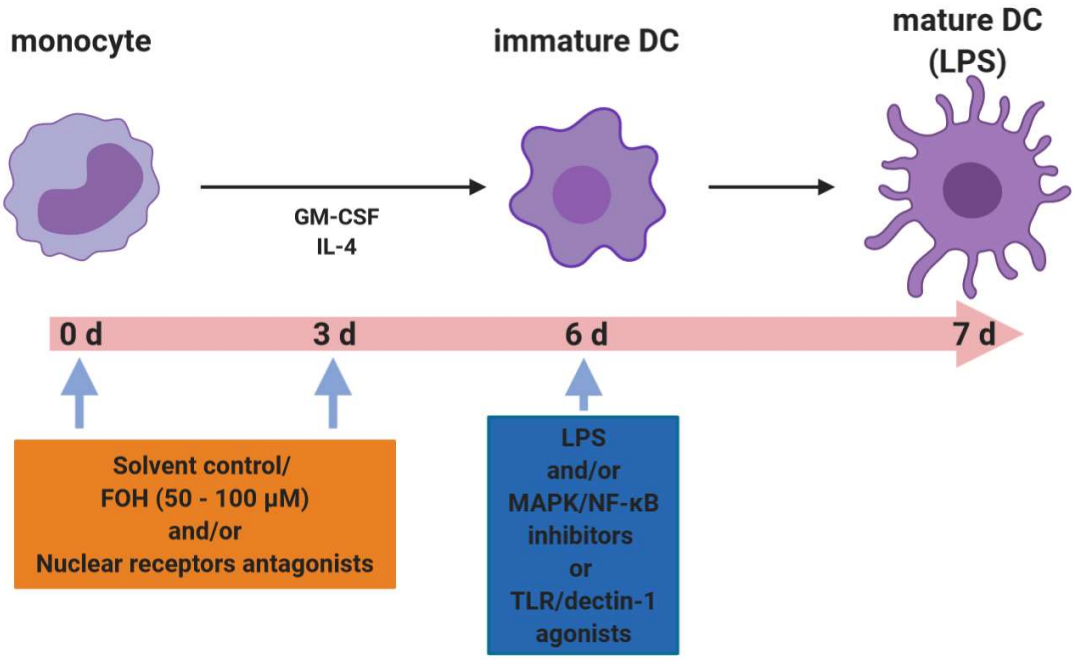

Figure 5. Generation of monocytes-derived DC

5.3 *Candida albicans* cultivation

Wild-type *C. albicans* strain SC5314 was maintained on YPD agar. For experiments, cells were transferred to M199 medium, pH 4 (9.8 g/l M199, 35.7 g/l HEPES, 2.2 g/l NaHCo3), and cultured at 30°C overnight to stationary phase. Germ tubes were induced by culturing in M199 medium, pH 8, for one hour at 37°C. Germ tubes were washed with HBSS and killed by adding 0.1 % Thimerosal (Sigma-Aldrich) for one hour at 37°C. MOI of 1 was used for confrontation assays.

5.4 Flow cytometry

All flow cytometric analyses were performed using the FACSCanto II (BD Bioscience) and the data was analyzed with FlowJo 7.6.4 software.

5.4.1 Viability determination

For determination of viability, cells were harvested and stained with Fixable Viability Stain V450 (BD Bioscience) for 15 min at room temperature prior cell surface staining. The percentage of viable cells (negative staining) was measured.

5.4.2 Characterization of cell surface markers

Phenotypic characterization of DC, iNKT, Th1 and Treg cells was performed by flow cytometry using fluorochrome-conjugated antibodies. 100 µl of cell suspension was stained with the following antibodies (See table 1). Isotype controls and fluorescence minus one (FMO) control were used for proper gating. After 20 min of staining, cells were resuspended in CellWash (BD Bioscience) and analyzed immediately on the flow cytometer.

Antibody/origin	Isotype/origin	Concentration
FITC anti-human CD14	Mouse IgG2a/Biolegend	100 µg/ml
PE anti-human CD1a	Mouse IgG1/Biolegend	50 µg/ml
PE anti-human CD80	Mouse IgG1/Biolegend	100 µg/ml
V450 anti-human CD86	Mouse IgG1/BD Bioscience	100 µg/ml
FITC anti-human CD40	Mouse IgG1/Biolegend	50 µg/ml
APC anti-human CD1d	Mouse IgG/Biolegend	100 µg/ml
PerCP anti-human HLA-DR	Mouse IgG2a/Biolegend	100 µg/ml
PerCp anti-human CD3	Mouse IgG1/Biolegend	50 µg/ml
PacificBlue anti-human CD4	Mouse IgG1/Biolegend	100 µg/ml
PE anti-human CD25	Mouse IgG1/Biolegend	25 µg/ml
APC anti-human CD85k (ILT3)	Mouse IgG1/Biolegend	100 µg/ml
FITC anti-human CD85j (ILT2)	Mouse IgG2b/Biolegend	100 µg/ml
PE anti-human CD85d (ILT4)	Mouse IgG2a/Biolegend	200 µg/ml
Brilliant Violet 421 anti-human CD274 (PD-L1)	Mouse IgG2b/Biolegend	50 µg/ml

PE anti-human TLR4	Mouse IgG2a/Biolegend	100 µg/ml
FITC anti-human 6B11 (anti-Vα24-Jα18)	Mouse IgG1/Biolegend	200 µg/ml

Table 1. Antibodies used for cell surface molecules and intracellular staining analysis

5.5 Invariant Natural Killer T cells expansion

Autologous T cells were collected by positive selection of $CD3^+$ cells with microbeads (Miltenyi Biotec) and stored in DMSO + 90%FBS at -80°C until used in co-culture experiments. Mature dendritic cells were generated from primary monocytes as described above (Section 5.2). At day 7, mDC were treated with 100 ng/ml of α-GalCer for 24 hours to obtain α-GalCer-loaded mDC and were co-cultured with autologous T cells in a 1:10 ratio ($1x10^5$ mDC : $1x10^6$ T cells) in 24-well plates. 24 hours later, 100 U/ml of recombinant human IL-2 (ImmunoTools) was added to induce T cell expansion. After 7 days, iNKT expansion was addressed by quantifying $CD3^+Vα24Jα18^+$ cells by flow cytometry. For cytokine reconstitution assays, recombinant human IL-12 (10 ng/mL) (ImmunoTools) and a mouse anti-human IL-10 blocking antibody (10 µg/mL) or a mouse IgG_{2B} isotype control (both obtained from R&D systems) were added from the first day of co-culture. At least $5x10^5$ events were acquired in the flow cytometer.

5.6 Intracellular staining

After 7 days of mDC – T cell co-culture, cells were collected and re-stimulated for 6 hours with PMA (50 ng/mL) and ionomycin (1 µg/mL), in presence of brefeldin A (5 µg/mL) and monensin (5 µg/mL) (Sigma-Aldrich). Intracellular staining of IFN-γ with FITC anti-human IFN-γ antibody (Biolegend, concentration: 25 µg/ml) and intracellular fixation and permeabilization buffer set according to the manufacturer´s protocol (eBioscience). To analyze Treg expansion, cells were fixed and permeabilized with the eBioscience FOXP3/Transcription factor staining set, followed by staining with FITC anti-human FOXP3 (Biolegend, concentration: 80 µg/ml). $CD3^+IFN-γ^+$ and $CD3^+CD25^+FOXP3^+$ cells were quantified by flow cytometry.

5.7 Phosphoflow

For experiments analyzing NF-κB and p38 MAPK phosphorylation, $5x10^5$ DC/well were seeded into 24-well plates and stimulated with 10 ng/ml LPS for 1 hour, 37°C and 5% CO_2. To stop the reaction, cells were fixed with intracellular fixation buffer (eBioscience) for 30 min at RT and permeabilized with 90% freezing methanol overnight. Cells were stained by using primary antibodies to phospho-NF-κB p65 (Ser536; clone 93H1) or phospho-p38 MAPK (Thr180/Tyr182; clone 12F8) (Cell signaling) for 1 hour in the dark at RT followed by staining with Dylight649-conjugated donkey anti-rabbit or Dylight488-conjugated donkey anti-mouse (Jackson ImmunoResearch) antibodies for 15 min at RT. PBS containing 0,5% BSA was used for washing and intracellular staining. Cells were analyzed in the flow cytometer.

5.8 Cytokine measurement

iDC were incubated in presence of either TLR and dectin-1 ligands for 24 hours, and then supernantants were collected and stored at -80 °C. Cytokine concentrations were analyzed with the ProcartaPlex immunoassay from ThermoFischer Scientific according to the manufacturer´s instructions.

5.9 RNA isolation and quantitative real-time PCR

Preparation of whole cell RNA was carried out with the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer´s instructions. NanoDrop (ThermoFisher) was used for the quantification of the RNA preparations. cDNA was generated from 25 ng of total RNA with the Precision nanoScript 2 Reverese transcription kit (Primer Design) and qRT-PCR was performed using the PrecisionPLUS MasterMix premixed with SYBRgreen kit (Primer Design) and Quantitect Primer Assay oligonucleotides (Qiagen) on a Stratagene Mx3500P cycler. Gene expression was normalized to the TBP reference gene and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

5.10 Statistical analysis

Data were analyzed with GraphPrism software version 6. Shapiro-Wilk normality test was used to assess Gaussian distribution. Comparison among groups was performed using a one-way ANOVA with Turkey's posttest for multiple comparisons. Otherwise, Kruskal-Wallis was used as a non-parametric test. Two-tailed unpaired Student´s *t* test was applied for comparison between two groups. $P < 0.05$ was considered as significant and marked in graphs using the following designations: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

6 Results

6.1 Viability of monocyte-differentiated DC in presence of farnesol

Farnesol (FOH) treatment inhibits proliferation while inducing apoptosis in several type of cells (51-57). Thus, Leonhardt *et al.* performed a viability analysis of iDC with Annexin-V and PI staining and showed that FOH treatment during the differentiation process did not influence the viability of iDC when used in concentrations up to 50µM, while retaining its effects on DC differentiation (56). To confirm this observation, viability of iDC generated in presence of FOH was investigated with the use of the fixable viability stain from BD. This dye binds to amines which are accessible in permeable cell membranes present in late-apoptotic/necrotic cells. As shown in figure 6, no differences could be observed in cell viability in iDC differentiated in presence of the solvent control or FOH (viable cells: mock-treated, 94 ± 5 %; with 100 µM FOH, 89 ± 7 %) when compared to untreated cells (94 ± 4 %). Since we observed no differences in viability for FOH concentrations 50 µM and 100 µM, subsequent experiments were performed using both FOH concentrations.

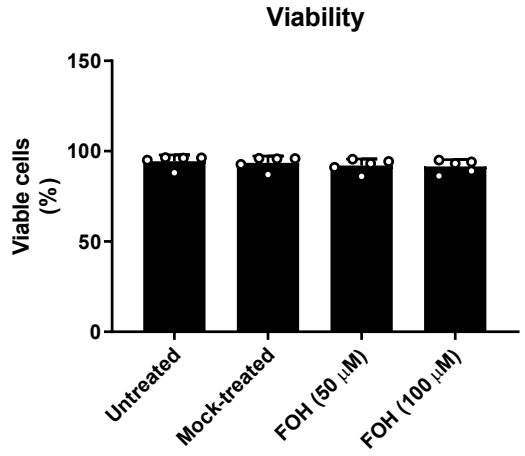

Figure 6. Viability of iDC differentiated in presence of FOH

Monocytes were differentiated into iDC in absence or presence of FOH (50 µM and 100 µM) and 1% methanol (mock-treated). Quantitative data of viable cells are shown as means ± SD from 4 independent experiments using cells from different donors.

6.2 Farnesol-differentiated DC have altered activation in response to distinct stimulation

Previous experiments performed by Leonhardt *et al.* showed that FOH impairs DC differentiation from monocytes by altering the surface molecule expression and the secretion of Th1-inducing cytokines (56). However, all the demonstrated effects of FOH on mDC so far were determined after stimulation of Toll-like receptor 4 (TLR4) signaling by LPS. Thus, FOH could be influencing DC maturation by changing the expression pattern of this PRR. Nevertheless, the presence of FOH throughout the differentiation and maturation process did not modify TLR4 surface expression (Figure 7). To get a broader view regarding the impact of FOH, DC were differentiated and stimulated with inactivated *C. albicans* germ-tubes and several agonists for Toll-like receptors and dectin-1. Similar to LPS stimulation, the expression of costimulatory CD40, CD80, CD86, and the MHC class II molecule HLA-DR were modulated when cells were exposed to FOH. Differentiation in presence of FOH impairs the expression of CD40 and CD80 after every stimulation tested. Moreover, CD86 surface exposure after LPS stimulation was reduced in FOH-instructed DC compared to the mock-treated cells (DC with 100 µM FOH, median of 2865 ± 515; mock-treated DC, median of 4589 ± 867, $P < 0.01$). Interestingly, CD86 surface expression was enhanced in FOH-differentiated DC when stimulated with Pam3CSK4 (TLR 1/2 agonist) and flagellin (TLR 5 agonists) (DC with 100 µM FOH: Pam3CSK4, median of 2286 ± 292, $P < 0.01$; flagellin, median of 2135 ± 265, $P < 0.001$) compared to mock-treated cells (mock-treated DC: Pam3CSK4, median of 1245 ± 547; flagellin, median of 819 ± 605). Furthermore, the MHC class II HLA-DR molecule was reduced in FOH-differentiated DC after stimulation with *C. albicans* (DC with 100 µM FOH: median of 1186 ± 585, mock-treated DC: median of 2410 ± 761, $P < 0.05$) (Figure 8).

In addition, the release of cytokines and chemokines was altered in FOH-differentiated DC in response to Toll-like receptor and dectin-1 ligands (Figure 9). Production of pro-inflammatory IL-8 and TNF-α was enhanced in FOH-differentiated DC when treated with Pam3CSK4 and flagellin compared to mock-treated cells. Furthermore, the release of the chemokine MCP-1 (CCL2) and the anti-inflammatory IL-1 receptor antagonist (IL-1RA) was increased in FOH-differentiated DC in response to every tested stimulus. Similarly, the secretion of anti-inflammatory IL-10 was enhanced in FOH-differentiated DC when treated

with Pam3CSK4 and flagellin (DC with 100 µM FOH: Pam3CSK4, 81 ± 72 pg/ml, $P < 0.001$; flagellin, 128 ± 124 pg/ml, $P < 0.05$) compared to mock-treated cells (mock-treated DC: Pam3CSK4, 13 ± 12 pg/ml; flagellin, 10 ± 6 pg/ml). Interestingly, the Th1-inducing cytokine IL-12 was reduced in FOH-differentiated DC stimulated with Toll-like receptors and dectin-1 ligands (DC with 100 µM FOH: LPS, 120 ± 174 pg/ml; $P < 0.001$; peptidoglycan, 11 ± 16 pg/ml; $P < 0.001$) compared to mock-differentiated DC (mock-treated DC: LPS, 3840 ± 1924 pg/ml; peptidoglycan, 2958 ± 1698 pg/ml). Altogether, these results show that FOH alters the differentiation and maturation of DC by modifying the surface expression of key molecules and the release of cytokines and chemokines in response to several stimuli, indicating that FOH-induced changes are independent of any specific PRR and FOH affects downstream signaling cascades.

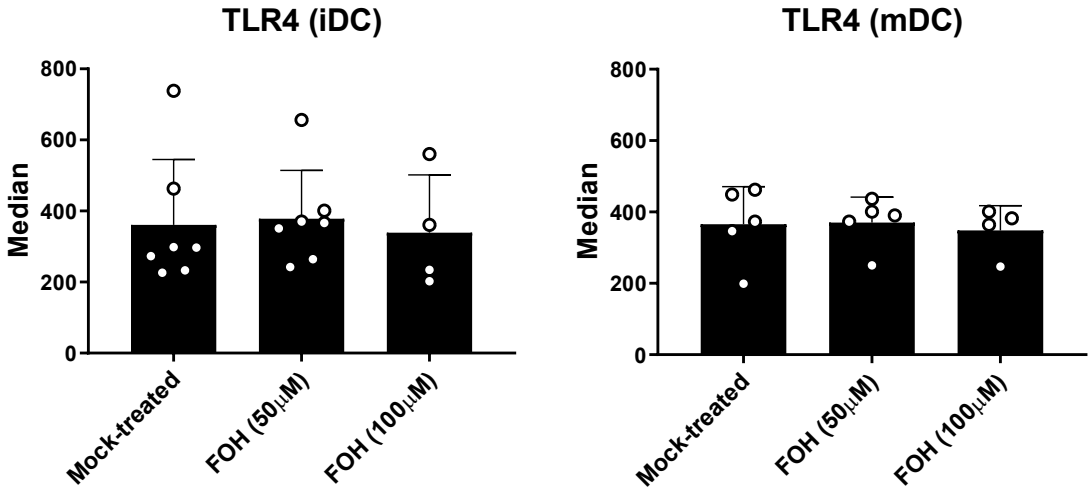

Figure 7. TLR4 expression in FOH-differentiated iDC and mDC

Monocytes were differentiated into iDC in the presence or absence of FOH and further stimulated with LPS for 24 hours. TLR4 median surface expression was quantified by flow cytometry. Quantitative data shown represent means ± SD from 4 independent experiments with cells isolated from different donors.

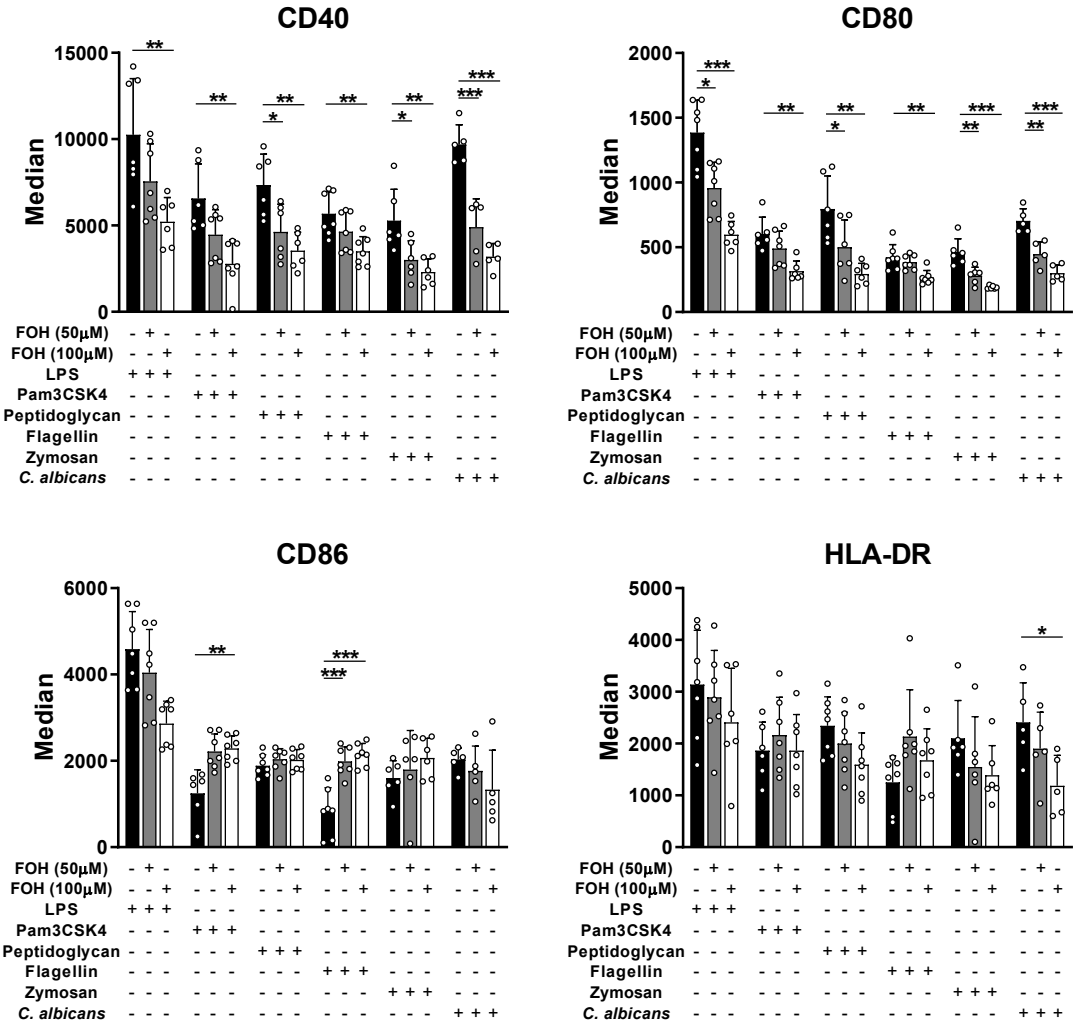

Figure 8. CD40, CD80, CD86 and HLA-DR expression in FOH-differentiated DC activated with distinct stimuli

Monocytes were differentiated into iDC in the presence or absence of FOH and further stimulated with either LPS (TLR4 agonist), Pam3CSK4 (TLR1/2 agonist), peptidoglycan (TLR2/6 agonist), flagellin (TLR5 agonist), Zymosan (Dectin-1 agonist) and *C. albicans* germ-tubes for 24 hours. For each surface marker, median surface expression was quantified by flow cytometry. Quantitative data shown represent means ± SD from 4 independent experiments with cells isolated from different donors (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

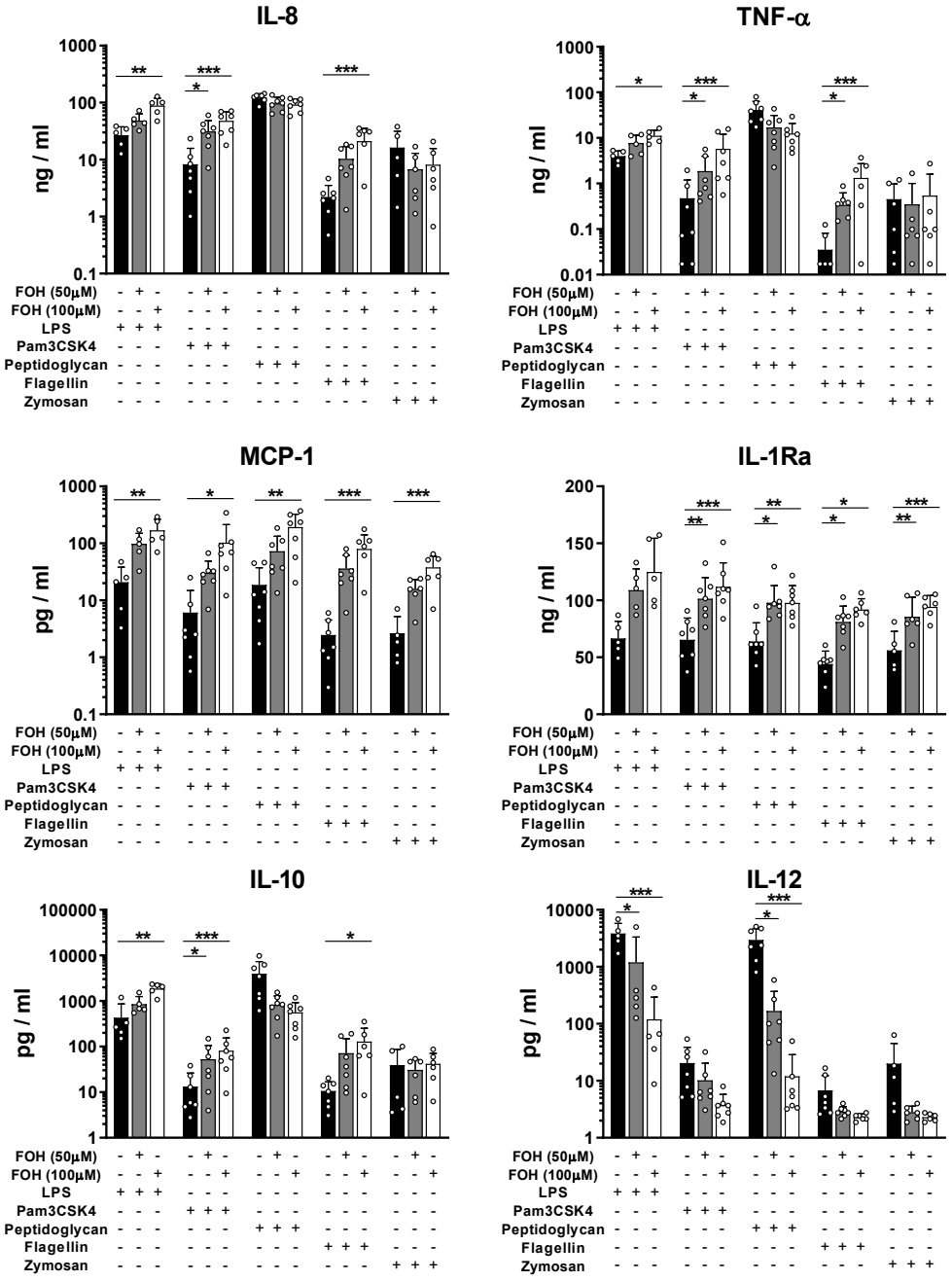

Figure 9. Cytokines and chemokines secretion of FOH-differentiated DC in response to distinct stimuli

Monocytes were differentiated into iDC in the presence or absence of FOH and further stimulated with either LPS (TLR4 agonist), Pam3CSK4 (TLR1/2 agonist), peptidoglycan (TLR2/6 agonist), flagellin (TLR5 agonist), Zymosan (Dectin-1 agonist), inactivated *C. albicans* for 24 hours. Cytokine concentrations were determined by multiplex assay. Data are means ± SD of 4 independent experiments using cells isolated from different donors (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

6.3 Molecular mechanisms modulated by farnesol on DC differentiation and maturation

6.3.1 Differentiation of DC in presence of farnesol increased the expression of genes involved in nuclear receptor activity

One of the most prominent effects in DC differentiated in presence of FOH was observed for the expression of CD1 transmembrane glycoproteins (56). FOH treatment shifts the expression pattern of CD1 molecules. While CD1a was not up-regulated in iDC and mDC, CD1d surface exposure was increased (Figure 10A). Consequently, the CD1a/CD1d ratio was reduced in FOH-differentiated DC (iDC with 100 µM FOH, ratio of 0.3 ± 0.1) compared to the mock-treated cells (mock-treated iDC: ratio of 106 ± 51, P <0.001) (Figure 10B). Since expression of CD1 molecules in DC is known to be tightly regulated by the nuclear receptors PPARγ and RARα (87, 156), screening of NR and their target genes was performed from the transcriptional data available from Leonhardt *et al.* (56). Several genes linked to NR activation, including target genes for PPARγ, RARα, and LXRα were upregulated during DC differentiation in presence of FOH (Figure 11A). Quantitative real time PCR was used to validate the expression of NR and selected target genes. In accordance with the microarray data, FOH induced the expression of *PPARγ* and its *bona fide* target gene *FABP4* as well as *LXRα* and its target gene *APOC1*. In addition, the RARα target gene *TGM2* was upregulated in FOH-differentiated DC. Nevertheless, the expression of the receptor did not change during differentiation with FOH. The effects of FOH was concentration-dependent and a higher expression of these genes was observed in cells differentiated with 100 µM FOH (Figure 11B).

These results confirmed FOH effects on CD1 molecules - while CD1a showed reduced expression, CD1d surface exposure was enhanced. Furthermore, FOH-differentiated DC have a higher expression of NR and their target genes.

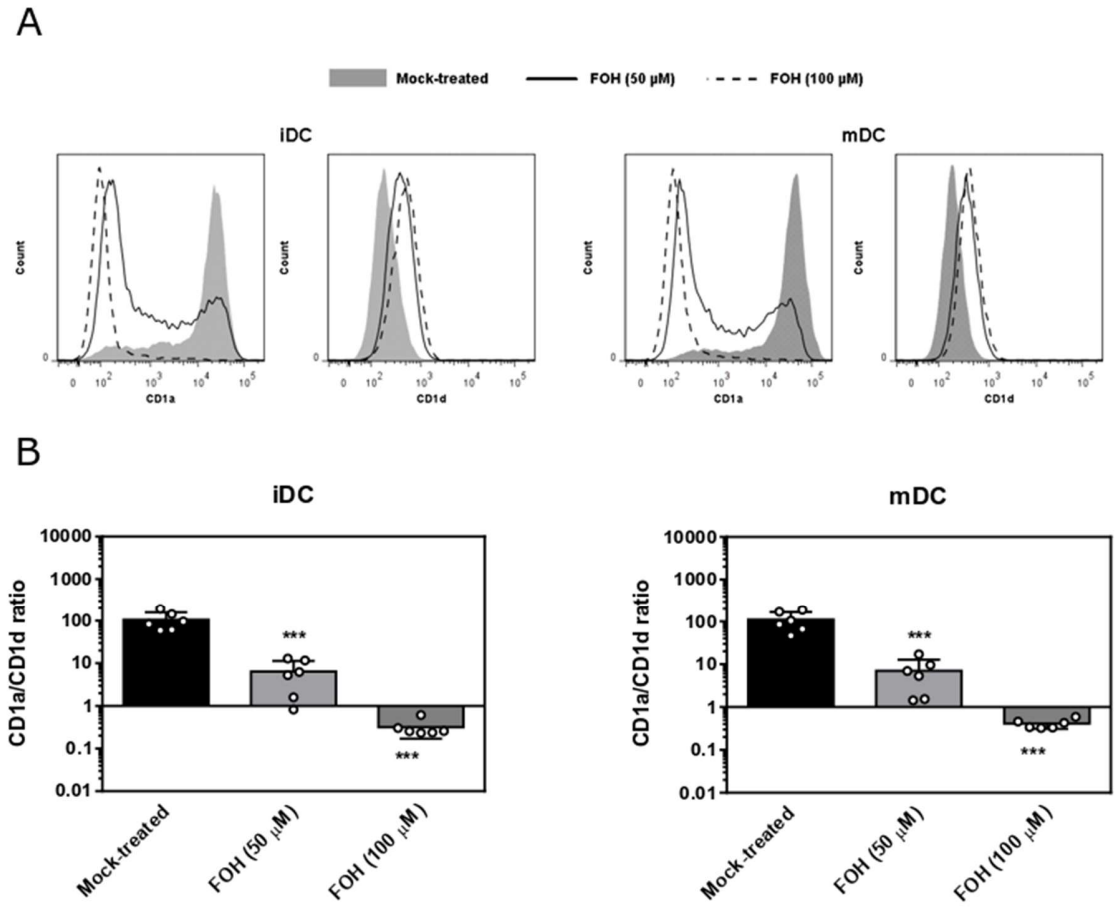

Figure 10. CD1 expression of DC differentiated in presence of FOH

Surface CD1 molecules expression of iDC and mDC was analyzed by flow cytometry. (A) Representative histogram after 6 days of differentiation (iDC) and following LPS stimulation for 24 hours (mDC). (B) Data shows the ratio between measured expression of CD1a and CD1d on DC differentiated with FOH (50 µM and 100 µM), compared to mock-treated DC. The bars show means ± SD from at least 4 independent experiments. (***P < 0,001, compared to mock-treated DC)

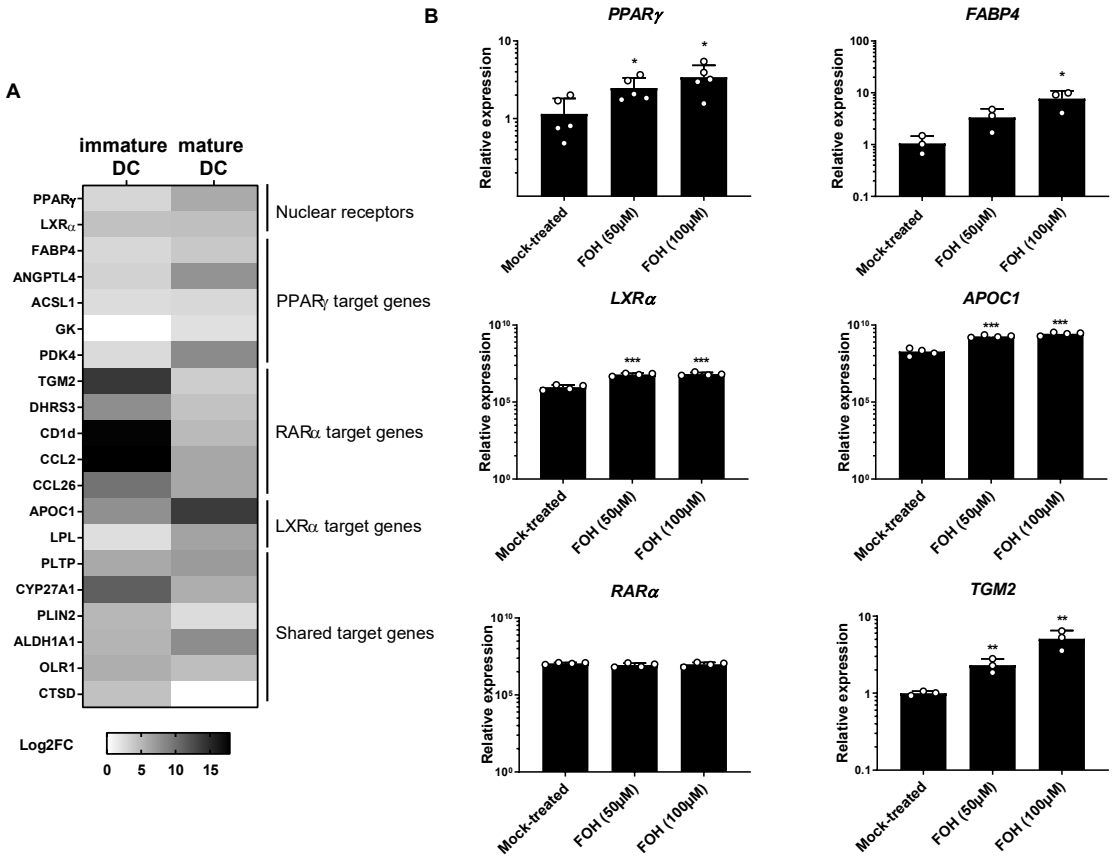

Figure 11. Expression of nuclear receptors and their target genes in DC-differentiated in presence of FOH.

(A) Microarray data of immature and mature DC differentiated in absence or presence of FOH (50 μM) was obtained from Leonhardt *et al.* and heat map of representative genes was generated. All displayed genes have a *P* value < 0.05 (56). (B) Confirmation of microarray results was performed by qRT-PCR. RNA was collected from iDC differentiated in presence or absence of FOH as described in experimental procedures. Gene expression was normalized to the TBP reference gene and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data are means ± SD from 3 independent experiments using cells from different donors (**P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared to mock-treated DC).

6.3.1.1 Activation of PPARγ and RARα leads to a DC phenotype comparable to farnesol-differentiated DC

To evaluate the role of NR activation in the immunophenotypic changes induced by FOH, cells were exposed to specific NR agonists and antagonists throughout the process of DC differentiation and analyzed for the surface expression of costimulatory and antigen-presenting molecules.

Previous reports have shown that activation of NR induce a similar immunophenotype to FOH-differentiated DC (87, 156). In agreement with this, the presence of FOH, RSG (PPARγ agonist) and AM (RARα agonist) impaired the expression of CD1a after the differentiation of monocytes into immature DC (iDC) and after 24 hours of LPS stimulation (mature DC, mDC) compared to mock-treated DC (mock-treated iDC, median of 20737 ± 11256, iDC with 100 µM FOH, median of 144 ± 62, $P < 0.001$; iDC with RSG, median of 157 ± 38, $P < 0.05$; iDC with AM, median of 274 ± 45, $P < 0.001$) (Figure 12). Interestingly, expression of CD1d was elevated in iDC and mDC in presence of FOH, RSG and AM compared to mock-treated cells (mock-treated iDC, median of 255 ± 51, iDC with 100 µM FOH, median of 638 ± 254, $P < 0.01$; iDC with RSG, median of 496 ± 85, $P < 0.05$; iDC with AM, median of 961 ± 393, $P < 0.001$). No effect was observed when cells were treated with LXRα agonist GW3965. Moreover, the expression of the costimulatory molecule CD80 was diminished in iDC and mDC treated with FOH, PPARγ and RARα agonists compared to mock-treated cells (mock-treated mDC, median of 1565 ± 390, mDC with FOH 100µM, median of 725 ± 212, $P < 0.001$; mDC with RSG, median of 951 ± 370, $P < 0.05$; mDC with AM, median of 819 ± 123, $P < 0.01$). In contrast, the expression of the costimulatory molecule CD86 was increased in iDC differentiated with FOH and agonists for PPARγ and RARα compared to mock-treated cells (mock-treated iDC, median of 139 ± 30, iDC with FOH 100µM, median of 732 ± 284, $P < 0.05$; iDC with RSG, median of 2334 ± 1265, $P < 0.001$; iDC with AM, median of 1181 ± 352, $P < 0.01$). No differences in CD86 expression were observed after LPS stimulation. These results showed that differentiation of DC in the presence of PPARγ and RARα agonists lead to a similar immunophenotype of DC compared to FOH-differentiated cells.

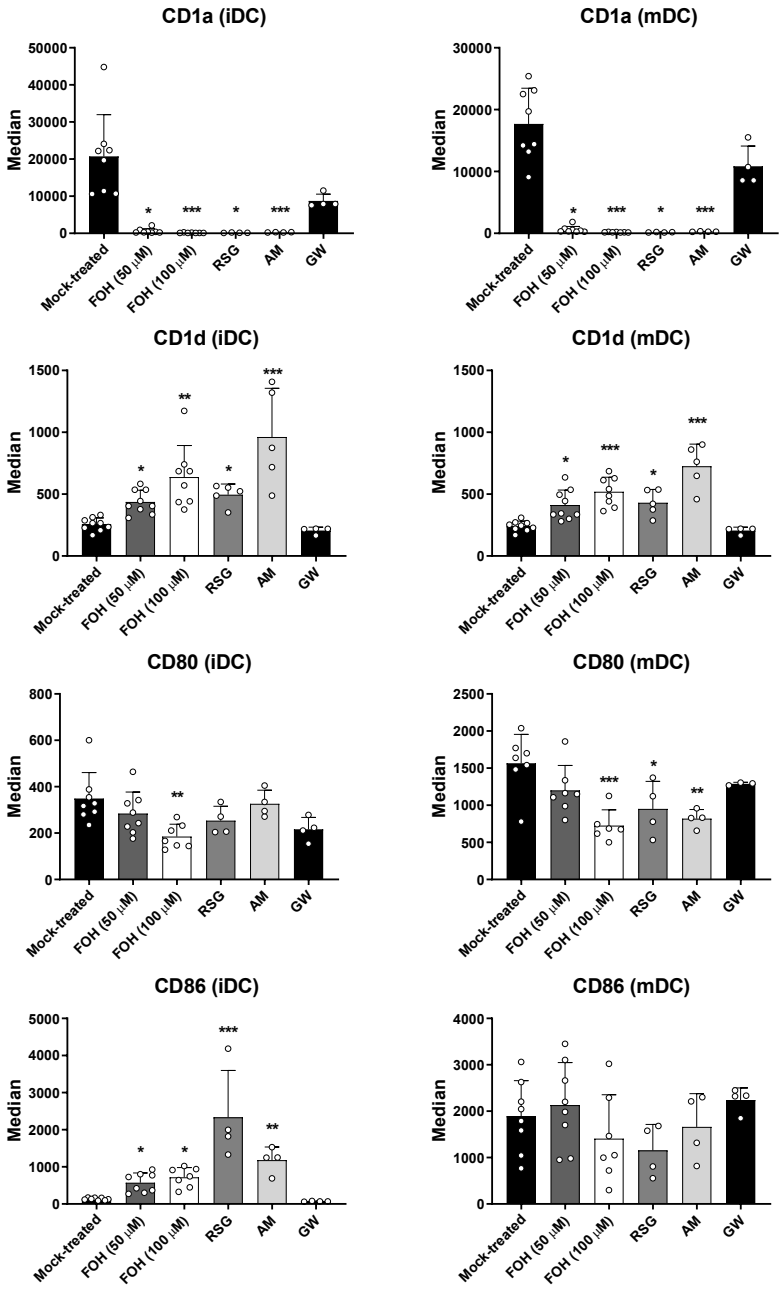

Figure 12. Surface molecules expression on DC differentiated in presence of farnesol and nuclear receptors agonists

Monocytes were differentiated into DC in the presence or absence of 50 µM or 100 µM FOH and nuclear receptors agonists. For each surface marker, median surface expression after 6 days of differentiation (iDC) and 24 hours stimulation with LPS (mDC) was quantified by flow cytometry. Quantitative data shown represent means ± SD from at least 3 independent experiments with cells isolated from different donors (**P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared to mock-treated DC). RSG: PPARγ agonist Rosiglitazone; AM: RARα agonist AM580; GW: LXRα agonist GW3965.

6.3.1.2 Nuclear receptor inhibitors have no significant effect on dendritic cell viability

DC were differentiated from monocytes in presence of previously published concentration of NR antagonists and viability was determined by flow cytometry. As shown in the figure 13, the presence or absence of FOH and NR antagonists during the differentiation process did not alter DC viability. Thus, subsequent experiments were performed by using these concentrations of FOH and NR antagonists.

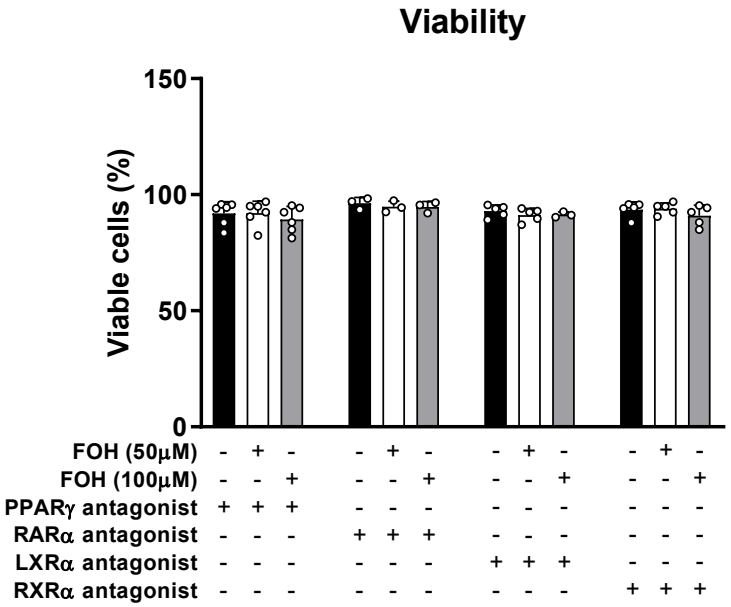

Figure 13. Viability of DC differentiated with nuclear receptor antagonists

Discrimination of viable from non-viable cells was evaluated by flow cytometry. Dead cells were identified by positive staining with the Fixable Viability Stain V450. Data are means ± SD from 3 independent experiments using cells isolated from different donors. PPARγ antagonist: GW9662, RARα antagonist: AGN193109, LXRα antagonist: GSK2033, RXR antagonist: HX531.

6.3.1.3 Farnesol modulates DC phenotype partially through activation of the PPARγ and RARα signaling pathways

DC generated in the presence of FOH were characterized by markedly lower surface expression of CD1a, but an increased expression of CD1d (Figures 10 and 12). to evaluate the role of nuclear receptor activation in the immunophenotypic changes induced by FOH, cells were exposed to various nuclear receptors antagonists during DC differentiation and analyzed for their surface expression of costimulatory and antigen-presenting molecules. The combination of FOH and NR antagonists were not enough to recover CD1a expression (Figure 14). In contrast, blocking the activity of PPARγ with the PPARγ antagonist GW9662 in iDC differentiated with 50 μM and 100 μM FOH returned CD1d expression to basal levels (mock-treated iDC, median of 266 ± 70; iDC with 50 μM FOH, median of 481 ± 145; iDC with 50 μM FOH and PPARγ antagonist, median of 265 ± 53). A similar return to basal CD1d expression levels DC was observed in the presence of RARα antagonist AGN193109 (mock-treated iDC, median of 289 ± 71; iDC with 50 μM FOH, median of 474 ± 149; iDC with 50 μM FOH and RARα antagonist, median of 243 ± 64). These effects were also maintained during the maturation process from iDC to mDC, suggesting that the increased CD1d surface exposure induced by FOH is regulated by a common pathway that involves both receptors. The use of LXRα antagonist affected neither CD1a nor CD1d surface expression during FOH treatment. However, blocking LXRα receptor activity downregulated CD1a expression (mock-treated iDC, median of 12731 ± 6598; with LXRα antagonist, median of 2845 ± 2469) and promoted the surface expression of CD1d (mock-treated iDC, median of 259 ± 85; with LXRα antagonist, median of 581 ± 203) in mock-treated control cells comparable to the effects induced by FOH (iDC with 50 μM FOH: CD1a, median of 255 ± 98; CD1d, median of 525 ± 227). To confirm the role of NR in the regulation of CD1 molecules by FOH, cells were differentiated in presence of an antagonist for RXRα, which serve as a partner for these receptors and are known to be essential for their functionality (127). Downmodulation of CD1a expression by FOH was not restored in presence of RXRα antagonist (Figure 15). Nevertheless, CD1d upregulation in FOH-instructed DC was blocked when the antagonist was present after the differentiation process (iDC with 100 μM FOH, median of 400 ± 81; with 100 μM FOH and RXRα antagonist, median of 241 ± 30, $P < 0.05$), reaching levels observed in mock-treated cells (mock-treated iDC, median of 202 ± 53).

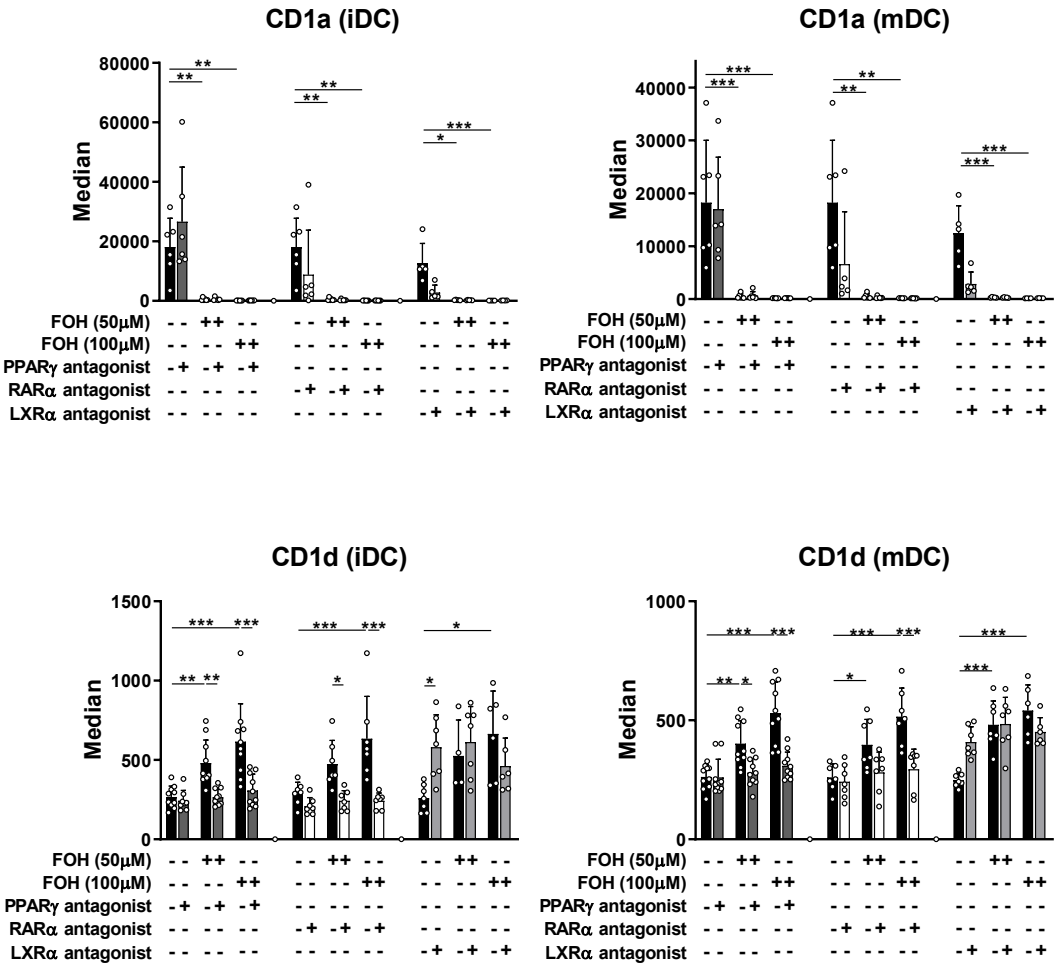

Figure 14. CD1 molecule expression induced by FOH in presence of nuclear receptor antagonists

Monocytes were differentiated into DC in the presence of either 50 µM or 100 µM FOH or mock treatment and the nuclear receptors antagonists indicated. For both surface markers, median surface expression after 6 days of differentiation (iDC) and 24 hours stimulation with LPS (mDC) was quantified by flow cytometry. Quantitative data shown represent means ± SD from at least 5 independent experiments with cells isolated from different donors (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). PPARγ antagonist: GW9662, RARα antagonist: AGN193109, LXRα antagonist: GSK2033, RXR antagonist: HX531.

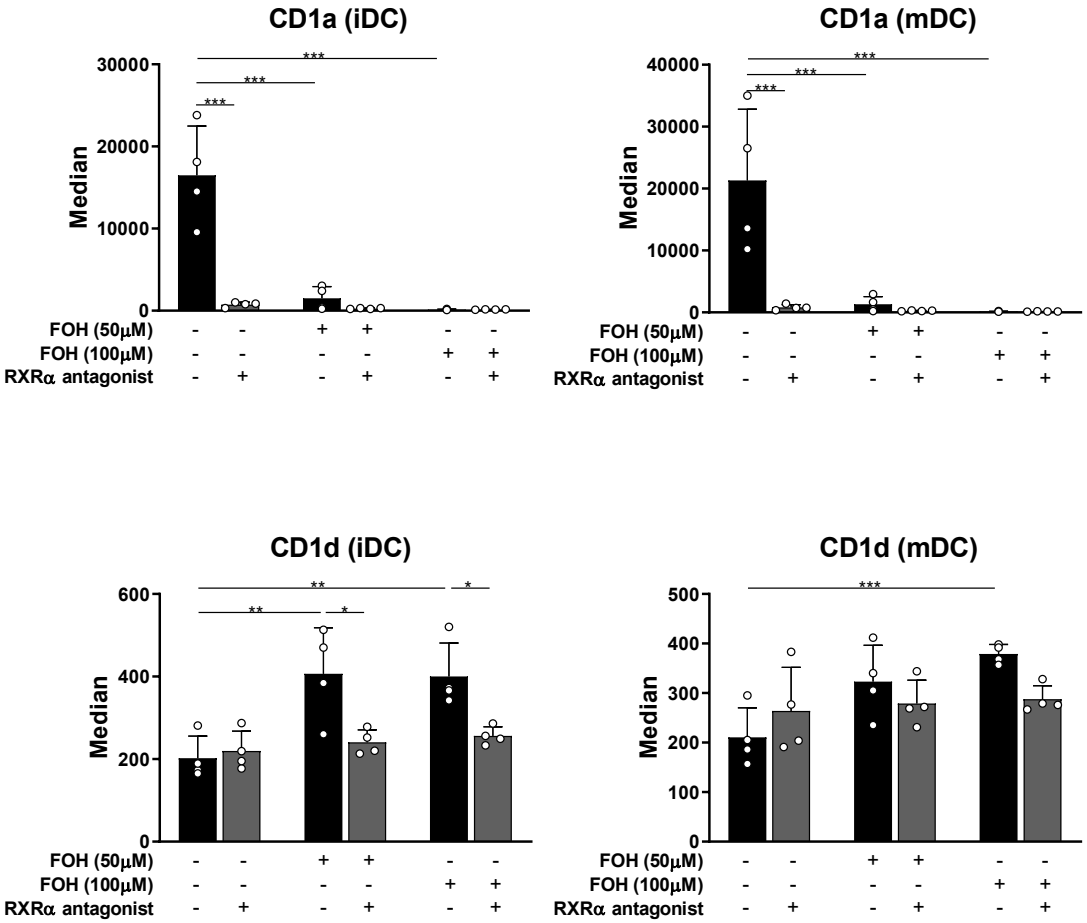

Figure 15. CD1 molecule expression induced by FOH in presence of RXRα antagonist

Monocytes were differentiated into DC in the presence of either 50 µM or 100 µM FOH or mock treatment and the RXR antagonist. For both surface markers, median surface expression after 6 days of differentiation (iDC) and 24 hours stimulation with LPS (mDC) was quantified by flow cytometry. Quantitative data shown represent means ± SD from 4 independent experiments with cells isolated from different donors (*P < 0.05; **P < 0.01; ***P < 0.001). RXRα antagonist: HX531.

To fully characterize the role of NR on the impact of FOH over DC differentiation and maturation, the expression of costimulatory and antigen-presenting molecules, which play a key role in DC functionality, was analyzed by flow cytometry. CD80 and CD40 expression was reduced in FOH-differentiated iDC and mDC (Figure 16). Nevertheless, the presence of PPARγ antagonist partially blocked CD80 downmodulation by lower FOH concentration in iDC (iDC with 50 μM FOH, median of 276 ± 65; iDC with 50 μM FOH and PPARγ antagonist, median of 454 ± 120, $P < 0.05$) and reached levels similar to mock-treated DC (median of 385 ± 140). In contrast, CD86 was upregulated in cells differentiated with FOH and the presence of PPARγ antagonist blocked this effect (mock-treated iDC, median of 202 ± 79, iDC with 50 μM FOH, median of 505 ± 207, iDC with 50 μM FOH and RARα antagonist, median of 242 ± 80, $P < 0.05$). Similarly, the presence of RARα antagonist blocked the upregulation of CD86 induced by FOH treatment (Figure 17). Interestingly, CD86 expression was partially down-modulated in presence of FOH after LPS stimulation and this effect was blocked during treatment with PPARγ antagonist. Finally, no significant differences were observed in HLA-DR expression after differentiation in presence of FOH. Nevertheless, HLA-DR expression was diminished in FOH-differentiated DC after LPS stimulation with no significant effects observed in presence of NR antagonists (mock-treated mDC: median of 3669 ± 213, mDC with 100μM FOH, median of 2360 ± 994, $P < 0.05$).

Altogether, these results confirmed alterations in the immunophenotype of DC differentiated in the presence of FOH. Even though altered expression of CD1a, CD40 and HLA-DR induced by FOH could not be restored by treatment with different NR antagonists, the altered expression of CD1d, CD80 and CD86 observed after differentiation was partially dependent on PPARγ and RARα activation.

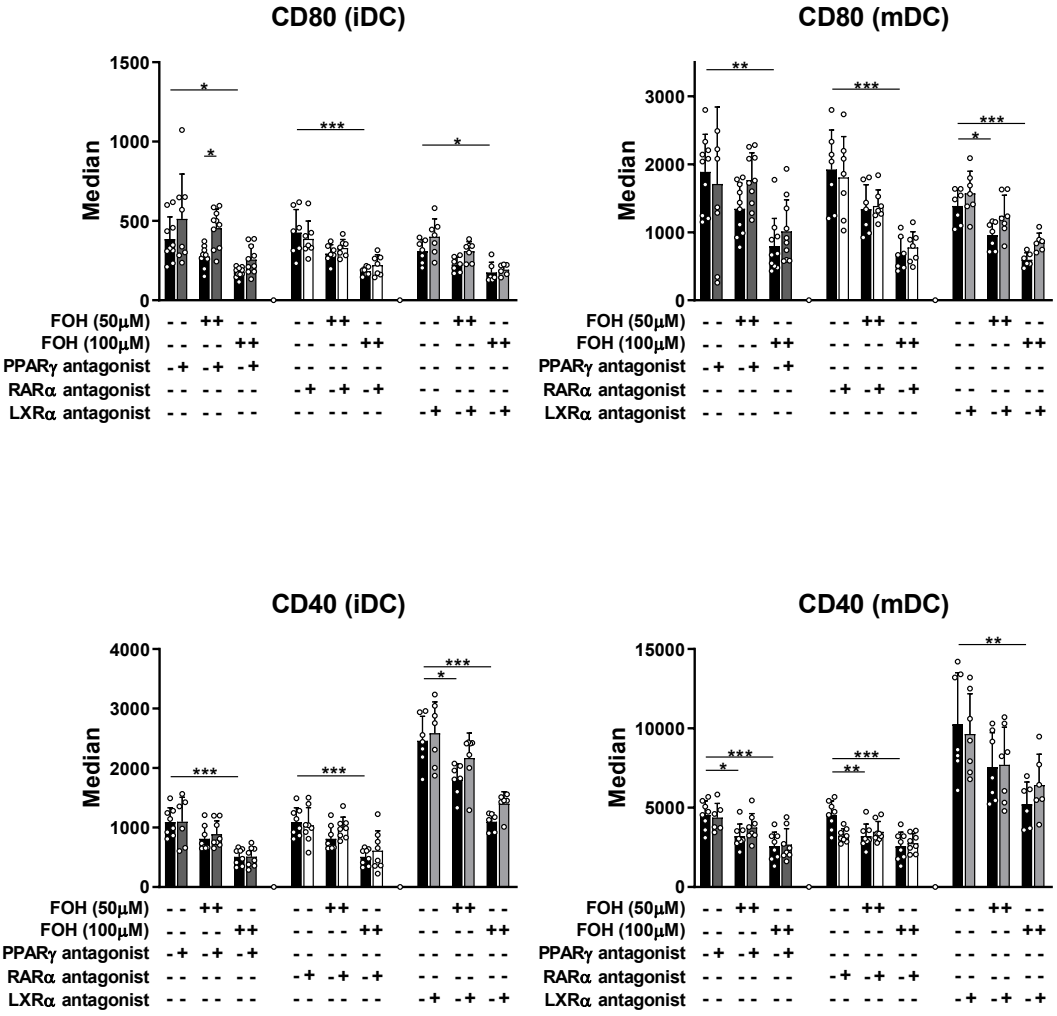

Figure 16. CD80 and CD40 molecule expression modulated by FOH in presence of nuclear receptors antagonists

Monocytes were differentiated into DC in the presence of either 50 µM or 100 µM FOH or mock treatment and the nuclear receptor antagonists indicated. For each surface marker, median surface expression after 6 days of differentiation (iDC) and 24 hours stimulation with LPS (mDC) was quantified by flow cytometry. Quantitative data shown represent means ± SD from at least 5 independent experiments with cells isolated from different donors (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). PPARγ antagonist: GW9662, RARα antagonist: AGN193109, LXRα antagonist: GSK2033.

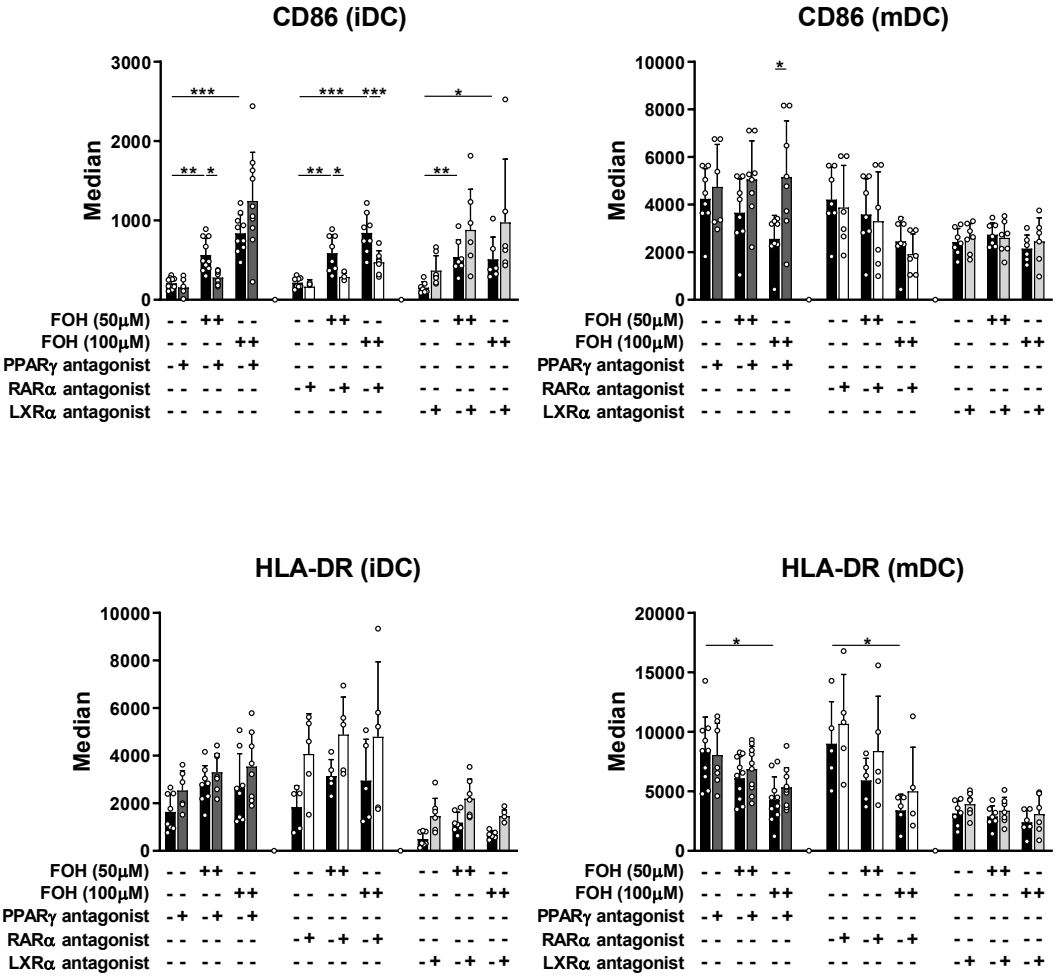

Figure 17. CD86 and HLA-DR molecule expression modulated by FOH in presence of nuclear receptors antagonists

Monocytes were differentiated into DC in the presence of either 50 µM or 100 µM FOH or mock treatment and the nuclear receptor antagonists indicated. For each surface marker, median surface expression after 6 days of differentiation (iDC) and 24 hours stimulation with LPS (mDC) was quantified by flow cytometry. Quantitative data shown represent means ± SD from at least 5 independent experiments with cells isolated from different donors (*P < 0.05; **P < 0.01; ***P < 0.001). PPARγ antagonist: GW9662, RARα antagonist: AGN193109, LXRα antagonist: GSK2033.

6.3.1.4 Release of cytokines and chemokines by farnesol-differentiated DC is mainly independent of nuclear receptors activation

The release of cytokines and chemokines by DC play an important role in regulating T cell activation. Cells were differentiated in presence or absence of NR antagonists and then stimulated with LPS to test the contribution of NR activity in the secretion of cytokines and chemokines (Figure 18). IL-8 and MCP-1 (CCL2) secretion was enhanced in FOH-differentiated DC and not influenced by NR antagonists. Interestingly, the FOH-induced increased secretion of pro-inflammatory TNF-α was blocked in presence of LXRα antagonist (100 µM FOH: without LXRα antagonist, 8.7 ± 3.5 ng/ml; with LXRα antagonist, 3.0 ± 1.4 ng/ml, $P < 0.05$) and restored TNFα levels to those observed in mock-treated DC (4.4 ± 1.0 ng/ml). In contrast, cells treated with RARα antagonist showed a reduction in TNF-α in every tested condition, suggesting a general and FOH-independent effect. Interestingly, differentiation of DC with either PPARγ, RARα or LXRα antagonists in the absence of FOH resulted in elevated levels of the anti-inflammatory cytokine IL-10, and synergistically increased its release in cells differentiated with 50 µM FOH. However, IL-10 release induced by higher concentration of FOH was not further enhanced by blocked NR activities. In contrast, the release of RANTES was dampened in FOH-instructed DC in a concentration-dependent manner and the presence of PPARγ antagonist partially increases the release of this chemokine in every tested condition. Moreover, IL-12 secretion was impaired in FOH-differentiated DC and treatment with the respective antagonists was not sufficient to restore the secretion of this cytokine by mDC. Treatment with the LXRα antagonist alone inhibited IL-12 secretion in mock-treated mDC and during exposure to the lower FOH concentration.

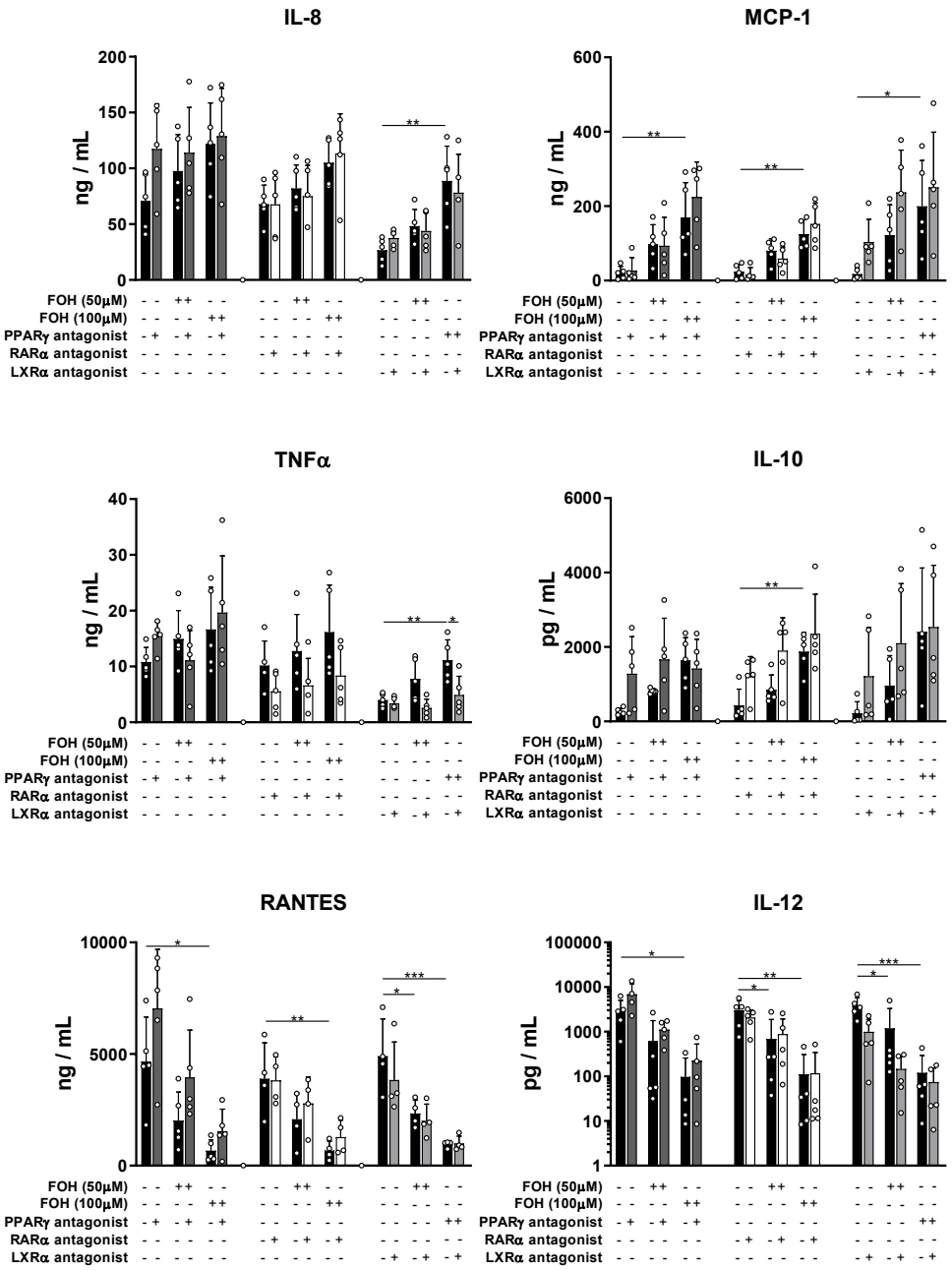

Figure 18. Cytokines and chemokines release by DC differentiated in presence of FOH and NR antagonists

Supernatants were collected after stimulation of iDC that were differentiated either in the absence or presence of FOH (50 µM and 100 µM) for 24 hours with LPS. Nuclear receptor activities were blocked using the respective antagonists: PPARγ antagonist GW9662; RARα antagonist AGN193109; LXRα antagonist GSK2033. Cytokine and chemokines concentrations were determined by multiplex assay. Data are means ± SD of 4 independent experiments using cells isolated from different donors (*P < 0.05; **P < 0.01; ***P < 0.001).

6.3.2 Role of MAPK and NF-κB signaling cascades in farnesol-differentiated DC

Pathway analysis of differentially expressed genes revealed that numerous processes were influenced by FOH stimulation, including antigen processing and presentation, PPAR signaling pathways, cytokine-cytokine interaction, and NF-κB signaling (56). In particular, activation of MAPK and NF-κB signaling pathways is known to be essential for proper differentiation and maturation of DC from monocytes (74, 75, 78, 79). To obtain further insights of the molecular mechanisms that regulate the altered maturation of DC, cell surface markers and cytokines release were measured from iDC treated with specific NF-κB and MAPK inhibitors prior stimulation with LPS.

6.3.2.1 MAPK and NF-κB signaling pathways modulate maturation of DC

Treatment of DC with MAPK and NF-κB inhibitors did not restore CD1a downregulation in FOH-differentiated DC (Figure 19). Interestingly, upregulation of CD1d by FOH was partially blocked in presence of p38 inhibitor (mDC with 100 µM FOH, median of 473 ± 84; mDC with 100 µM FOH and p38 inhibitor, median of 341 ± 52, *p*: 0.07). As expected, the upregulation of CD80 by LPS stimulation was significantly blocked in the presence of p38 MAPK and NF-κB inhibitors (mock-treated mDC, median of 1102 ± 165; with NF-κB inhibitor, median of 778 ± 171, $P < 0.01$; with p38 inhibitor, median of 645 ± 100, $P < 0.001$). Moreover, during FOH treatment we observed a synergistic reduction on the expression of CD80 in the presence of p38 MAPK and NF-κB inhibitors (mDC with 50 µM FOH, median of 810 ± 168; mDC with 50 µM FOH and NF-κB inhibitor, median of 559 ± 150, $P < 0.05$; mDC with 50 µM FOH and p38 inhibitor, median of 409 ± 86, $P < 0.001$). In contrast, CD40 and CD86 surface exposure in DC-differentiated in absence or presence of FOH was blocked only in the presence of the NF-κB inhibitor. Finally, the surface exposure of HLA-DR was not affected in any of the conditions tested.

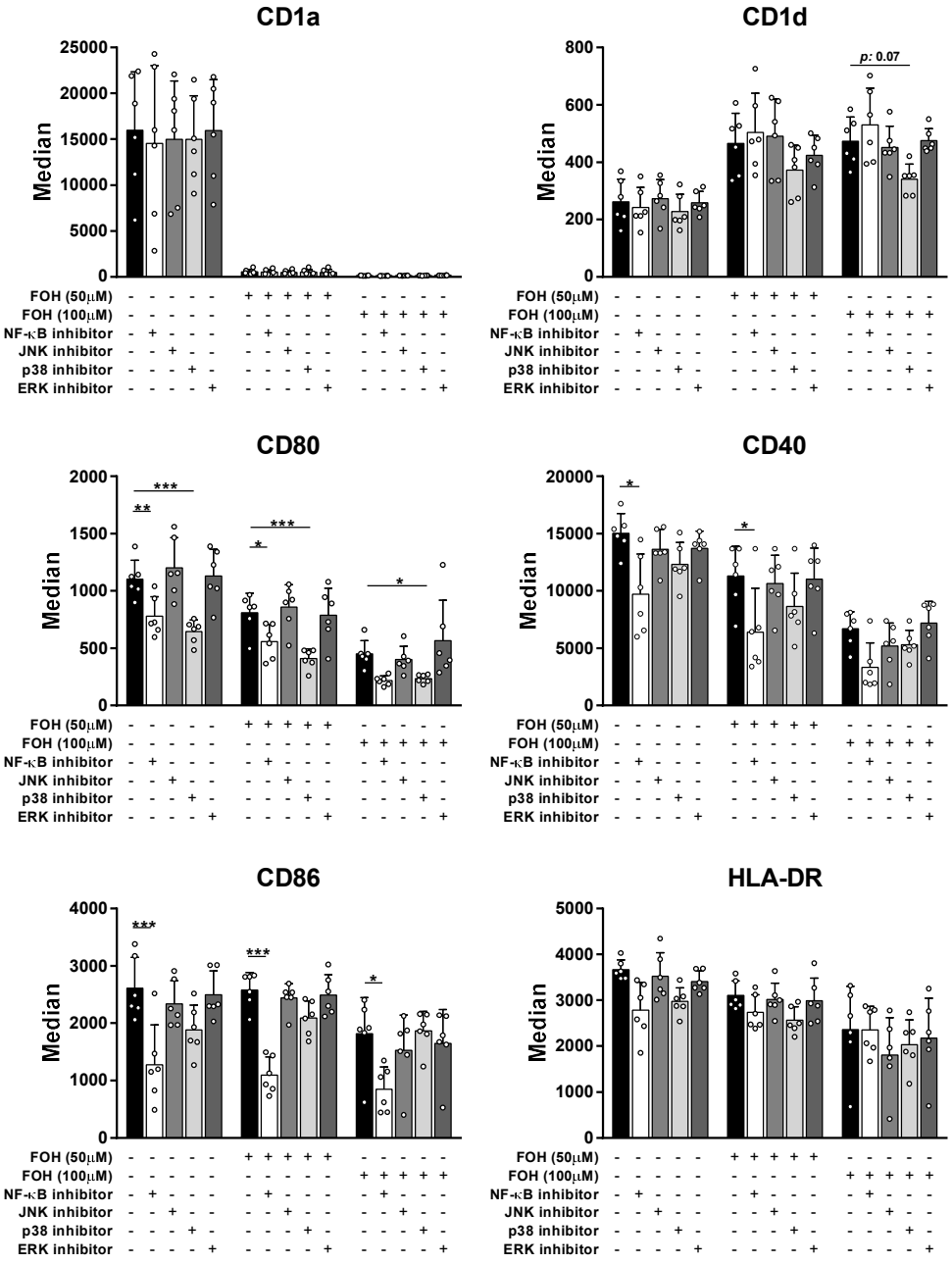

Figure 19. Surface molecule expression in FOH-differentiated mDC in presence of MAPK and NF-κB inhibitors

Monocytes were differentiated into iDC in the presence of either 50 µM or 100 µM FOH or mock treatment followed by stimulation with LPS for 24 hours. For each surface marker, median surface was quantified by flow cytometry. Quantitative data represent means ± SD from 3 independent experiments with cells isolated from different donors (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). MAPK and NF-κB activation were blocked using respective antagonists: SC75741: NF-κB inhibitor, SP600125: JNK inhibitor, SB203580: p38 inhibitor, FR180204: ERK1/2 inhibitor.

6.3.2.2 Farnesol regulates cytokine and chemokine secretion through MAPK and NF-κB signaling pathways

Our previous results showed that FOH modulate the expression CD1d and costimulatory molecules through activation of NF-κB and p38 MAPK. Thereby, we proceeded our analysis by measuring the release of cytokines and chemokines in DC differentiated in presence or absence of FOH, NF-κB and MAPK inhibitors. Even though the release of IL-8 was not blocked by any of the inhibitors tested (Figure 20), the presence of NF-κB inhibitor blocked the enhanced release of MCP-1, TNF-α and IL-10 induced by FOH. Interestingly, treatment with JNK and p38 MAPK inhibitors impaired the elevated secretion of TNF-α and IL-10 induced by FOH, respectively. Moreover, reduction in RANTES secretion was observed in presence of NF-κB, JNK and p38 inhibitors in every tested condition. Similarly, NF-κB and p38 signaling pathways also regulated IL-12 release in mock-treated mDC (mock-treated mDC, 3217 ± 1379 pg/ml; mock-treated mDC with NF-κB inhibitor, 1206 ± 796 pg/ml, $P < 0.05$; mock-treated mDC with p38 inhibitor, 61 ± 24 pg/ml, $P < 0.001$). In contrast, during FOH treatment a synergistic reduction was observed in the presence of the p38 inhibitor (50 μM FOH, 402 ± 221 pg/ml; 50 μM FOH and p38 inhibitor, 27 ± 13 pg/ml). To confirm if FOH treatment modify the activation of these signaling pathways, phosphorylation levels of p65-NF-κB and p38 MAPK was evaluated through flow cytometry. PhosphoFlow analysis showed that iDC differentiated in the presence of FOH have a higher activation of NF-κB compared to mock-treated DC (0 min with 100 μM FOH, fold change of 3 ± 0.3; without 100 μM FOH, fold change of 2.5 ± 0.4, $P < 0.05$) (Figure 21). As expected, phosphorylation of p65 increased after 60 min of LPS stimulation in mock-treated DC, but no differences were observed compared to FOH-differentiated DC. Finally, DC differentiated in presence of 100 μM FOH showed an enhanced p38 phosphorylation compared to mock-treated DC before and after 60 min of LPS stimulation (Figure 22). Altogether, these results indicate that FOH modulates activation of MAPK and NF-κB signaling pathways which results in altered expression of costimulatory molecules and cytokine release.

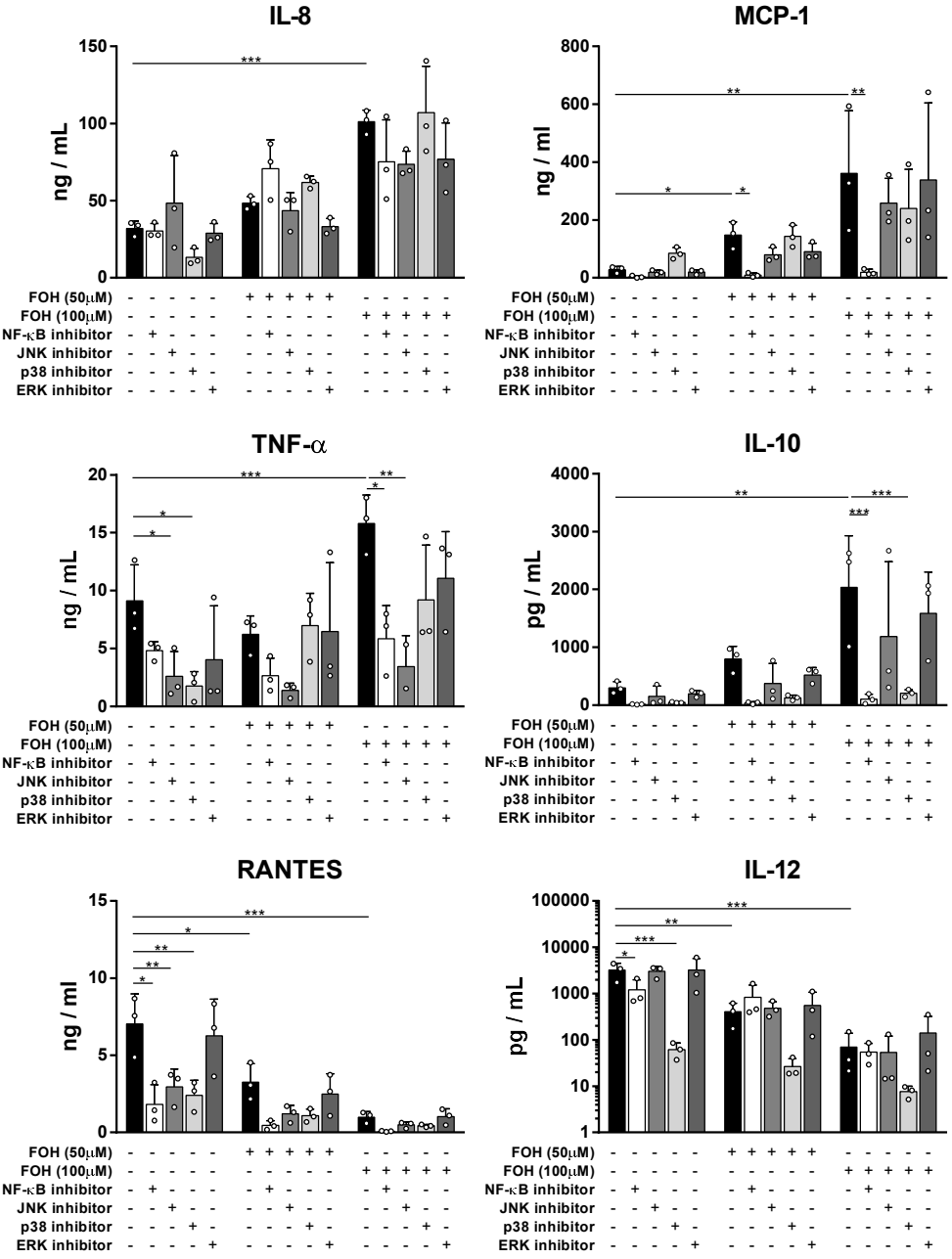

Figure 20. Cytokine and chemokine release by DC differentiated in presence of FOH and NF-κB and MAPK pathway inhibitors

iDC were differentiated either in the absence or presence of FOH (50 µM and 100 µM), stimulated with LPS for 24 hours, and then supernatants were collected. MAPK and NF-κB activation were blocked using respective antagonists: SC75741: NF-κB inhibitor, SP600125: JNK inhibitor, SB203580: p38 inhibitor, FR180204: ERK1/2 inhibitor. Cytokine concentrations were determined by multiplex assay. Data are means ± SD of 3 independent experiments using cells isolated from different donors (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

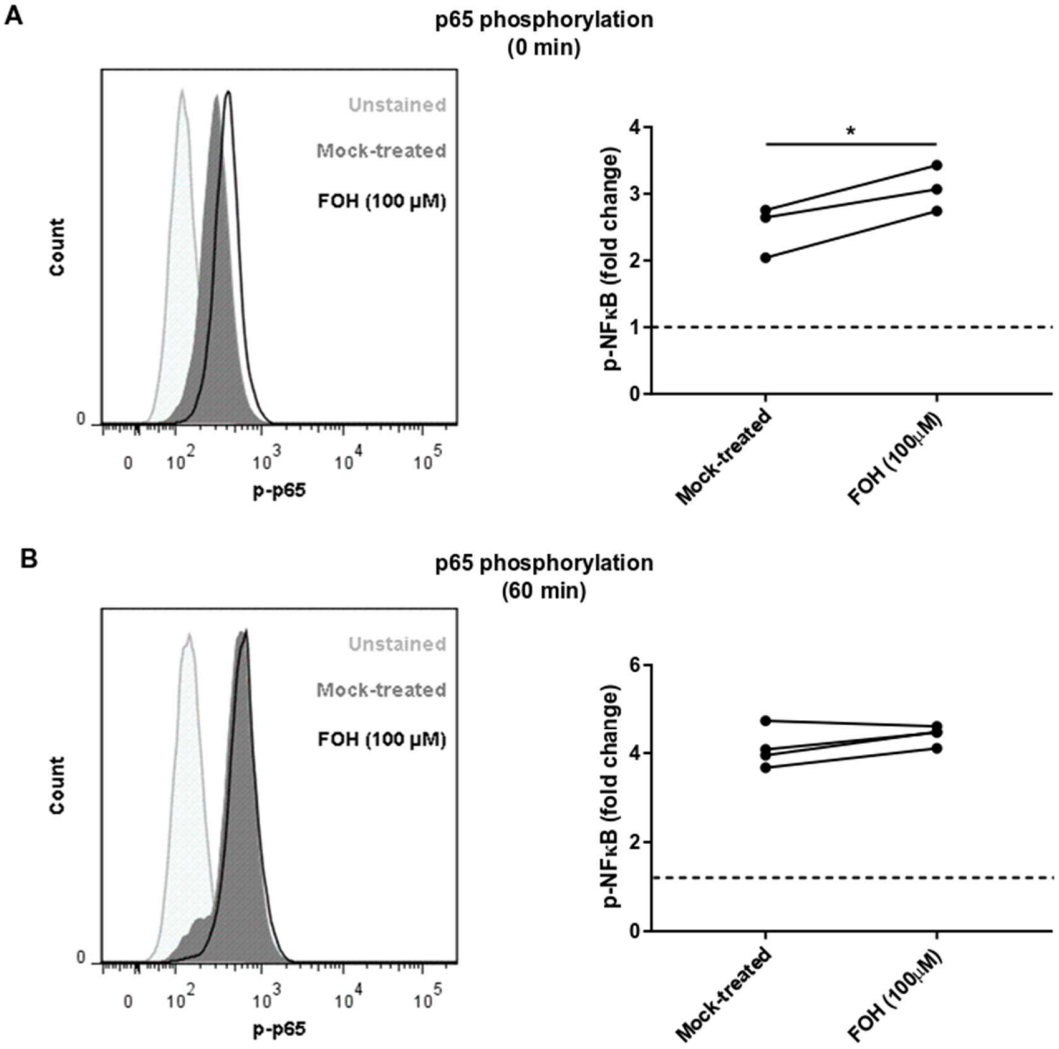

Figure 21. Analysis of NF-κB activation in FOH-differentiated mDC

iDC were differentiated either in the absence (mock-treated) or presence of FOH (100 µM) and phosphorylation of p65 NF-κB was addressed through flow cytometry. Figure shows representative histogram of p38 phosphorylation followed by statistical analysis of DC before (A) and after 60 min of LPS stimulation (B). Quantitative analysis is from 4 independent experiments with cells isolated from different donors and shown as fold change values acquired between DC-differentiated in absence or presence of FOH and normalized to values observed in unstained DC (*$P < 0.05$).

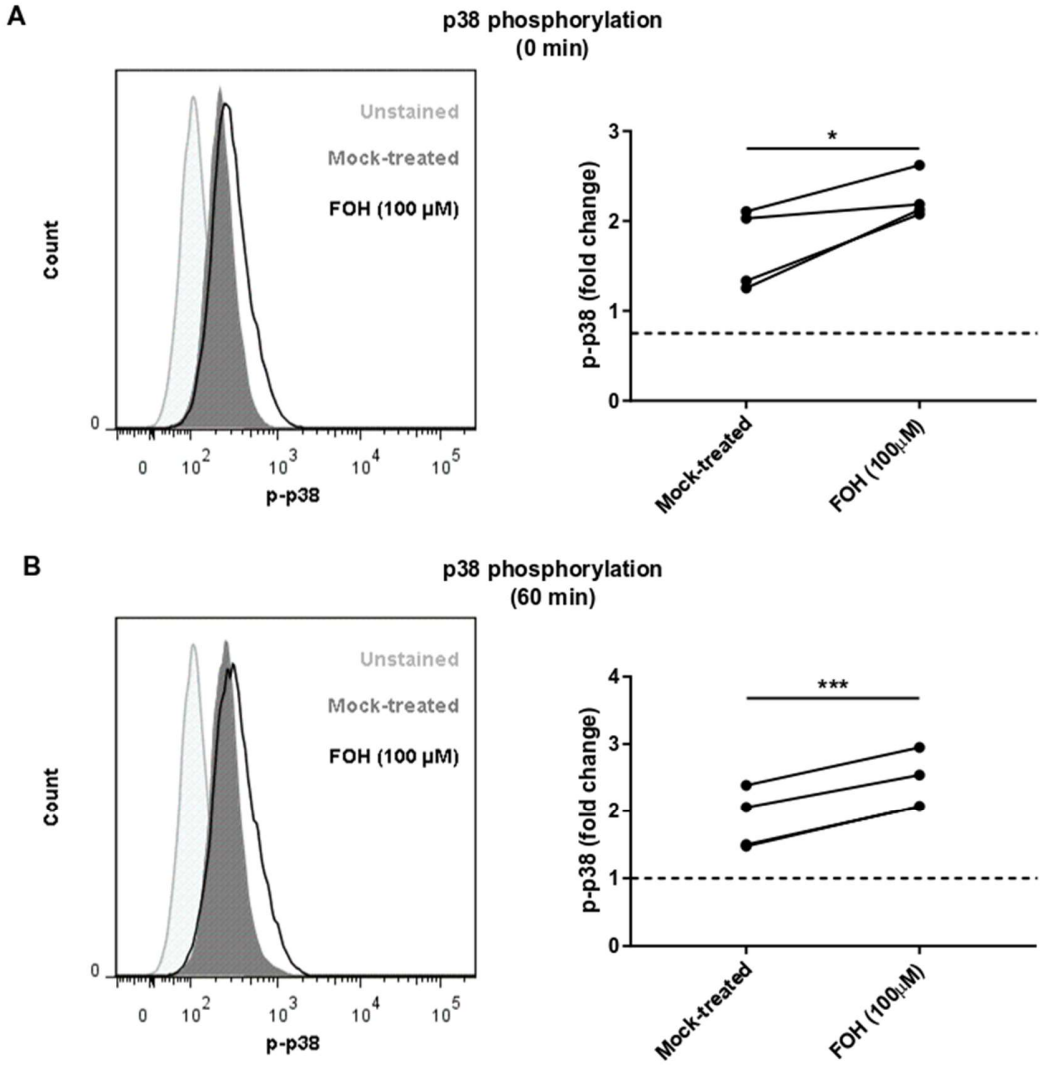

Figure 22. Analysis of p38 activation in FOH-differentiated mDC

iDC were differentiated either in the absence (mock-treated) or presence of FOH (100 µM) and phosphorylation of p38 MAPK was addressed through flow cytometry. Figure shows representative histogram of p38 phosphorylation followed by statistical analysis of DC before (A) and after 60 min of LPS stimulation (B). Quantitative analysis is from 4 independent experiments with cells isolated from different donors and shown as fold change values acquired between DC-differentiated in absence or presence of FOH and normalized to values observed in unstained DC (*$P < 0.05$).

6.4 Functional impact of farnesol treatment in DC

DC play an important role in orchestrating immune response through antigen-presentation and activation of effector T cells (65). Consequently, alteration of these functions might have important consequences in the ability to overcome infection by pathogenic microorganisms. To evaluate if FOH treatment alters DC capacity to activate T cells, mDC and T cell co-cultures were performed and the ability of mDC to activate iNKT, Th1 and Treg was adressed by flow cytometry.

6.4.1 Farnesol-differentiated DC have a lower capacity to activate iNKT and Th1 cells

The previous results showed that FOH alters dramatically the expression of CD1d molecules through PPARγ, RARα and partially through p38 MAPK activation. CD1d is a major-histocompatibility-complex (MHC) class-1-like molecule that presents lipid antigens to invariant Natural Killer T (iNKT) cells to activate them (85, 87). To test whether the elevated CD1d surface levels induced by FOH would correlate with an increased capability of mDC to induce iNKT activation, expansion of iNKT cells was analyzed by flow cytometry. Surprisingly, iNKT proliferation was reduced in the presence of FOH-differentiated mDC (50 µM FOH, fold change of 2.5 ± 1.2, $P < 0.01$) compared to the mock-treated mDC (Fold change of 7.3 ± 1.7), regardless of their significantly enhanced CD1d levels (Figure 23). Although CD1d expression induced by FOH was dependent on both PPARγ and RARα activity, blocking NR activities resulted in comparably low iNKT expansion.

All data reported so far suggest that FOH-differentiated mDC have a defect to induce proper T cell responses, by reducing the expression of costimulatory molecules and the MHC class II molecule HLA-DR. To evaluate if FOH alters the capacity of mDC to activate Th1 cells, mDC differentiated in presence or absence of FOH and NR antagonists were co-cultured with autologous T cells for 7 days and IFN-γ production was analyzed by flow cytometry after restimulation with PMA/ionomycin. As expected, T cells co-cultured with mock-treated mDC (Fold change of 16.6 ± 5.4) have a higher ability to induce IFN-γ production compared to FOH differentiated mDC (50 µM FOH, fold change of 5.2 ± 2.7, $P < 0.05$) (Figure 24). The presence of specific antagonists for PPARγ and RARα did not restore the capability of mDC to induce IFN-γ production by T cells.

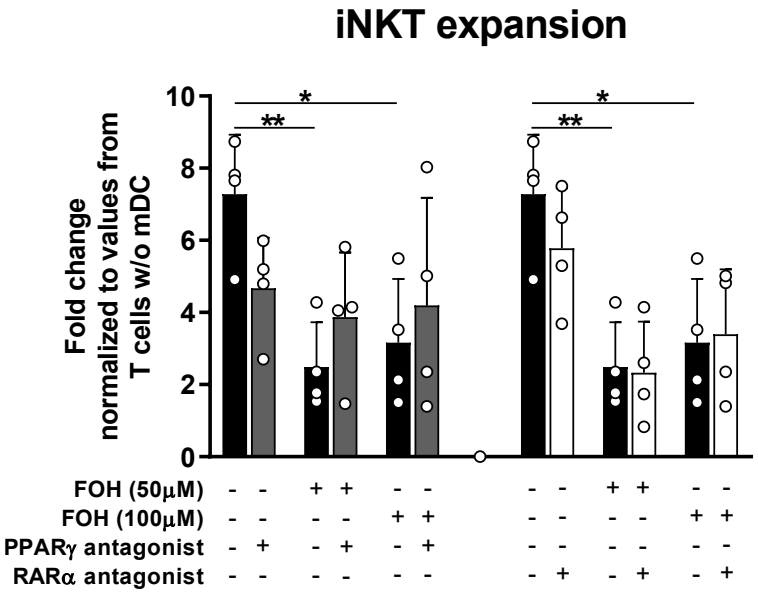

Figure 23. iNKT expansion by FOH treated-DC

Autologous T cells were co-cultured with mDC for 7 days. iNKT expansion was measured by flow cytometry and quantitative analysis from 4 independent experiments with cells isolated from different donors is displayed. Quantitative analysis is shown as means ± SD of fold change values acquired between % iNKT cells of total T cells in co-culture with mock- or FOH-treated mDC normalized to values observed in T cells cultured without mDC (*P < 0.05; **P < 0.01). GW: PPARγ antagonist GW9662; AGN: RARα antagonist AGN193109.

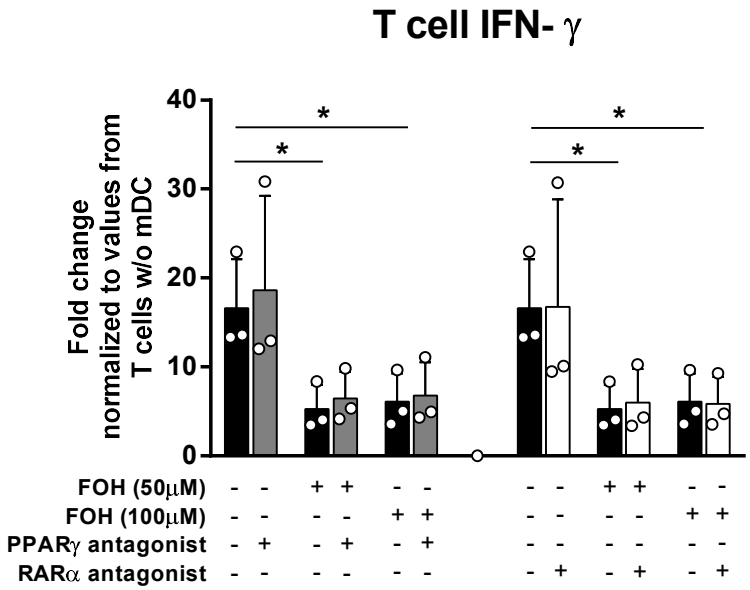

Figure 24. IFN-γ production by T cells co-cultured with mDC

Autologous T cells were co-cultured with mDC for 7 days. T cells were collected and re-stimulated with PMA (50 ng/mL) and ionomycin (1 µg/mL), in the presence of brefeldin A (5 µg/mL) and monensin (5 µg/mL) for 6 hours. IFN-γ production was analyzed by intracellular staining and flow cytometry. Quantitative analysis of 3 independent experiments is shown as means ± SD of fold change values acquired between % IFN-γ$^+$ cells of total T cells co-culture with mock- or FOH-treated mDC normalized to values observed in T cells cultured without mDC (*P < 0.05; **P < 0.01). PPARγ antagonist: GW9662; RARα antagonist: AGN193109.

6.4.1.1 Altered secretion of IL-12 and IL-10 influences the capacity of farnesol-differentiated DC to activate different T cell subsets

Although FOH increased CD1d expression in DC, no positive effect was observed in their capacity to induce iNKT expansion. A possible explanation could be the increased IL-10 secretion and reduced IL-12 release observed in FOH-treated mDC. Due to their potent effect on activating T cells, IL-12 family members are an important link between innate and adaptive immunity. Moreover, IL-10 is known to inhibit IL-12 production by macrophages and DC (206). To test if the shift in the IL-12/IL-10 ratio observed in FOH-differentiated DC might play a role in iNKT activation (Figure 25A), T cell-mDC co-culture experiments were performed in the presence of increased IL-12 levels by addition of recombinant IL-12, while IL-10 activity was reduced by the use of blocking antibody. Interestingly, the reduced iNKT proliferation observed in presence of FOH-differentiated DC was increased after reconstitution of the IL-12/IL-10 milieu by addition of recombinant IL-12 and neutralizing αIL-10, reaching levels similar to the mock T cell-mDC co-culture (mock-treated mDC, fold change of 10.1 ± 3.7, with 50 µM FOH, fold change of 4.7 ± 2.9; 50 µM FOH and IL-12/αIL-10, fold change of 12.1 ± 4.8, $P < 0.05$) (Figure 25B). Furthermore, addition of recombinant IL-12 and αIL-10 also enhanced iNKT proliferation in mock T cell-mDC co-culture (Fold change of 15.4 ± 3.8).

To test if the altered IL-12 and IL-10 release also played a role in mDC capability to induce IFN-γ production by T cells, co-culture of mDC and autologous T cells for 7 days was performed and IFN-γ production by T cells was induced by restimulation with PMA/ionomycin (Figure 26). In line with the result observed for iNKT expansion, the reduced percentage of IFN-γ producing T cells co-cultured with FOH-differentiated mDC was restored to levels observed in mock-treated mDC co-culture (Fold change of 6.8 ± 4.9) when elevated concentrations of IL-12 and IL-10 blocking antibody were present (50 µM FOH, fold change of 1.3 ± 0.3; 50 µM FOH and IL-12/αIL-10, fold change of 6.7 ± 4.8, $P < 0.05$).

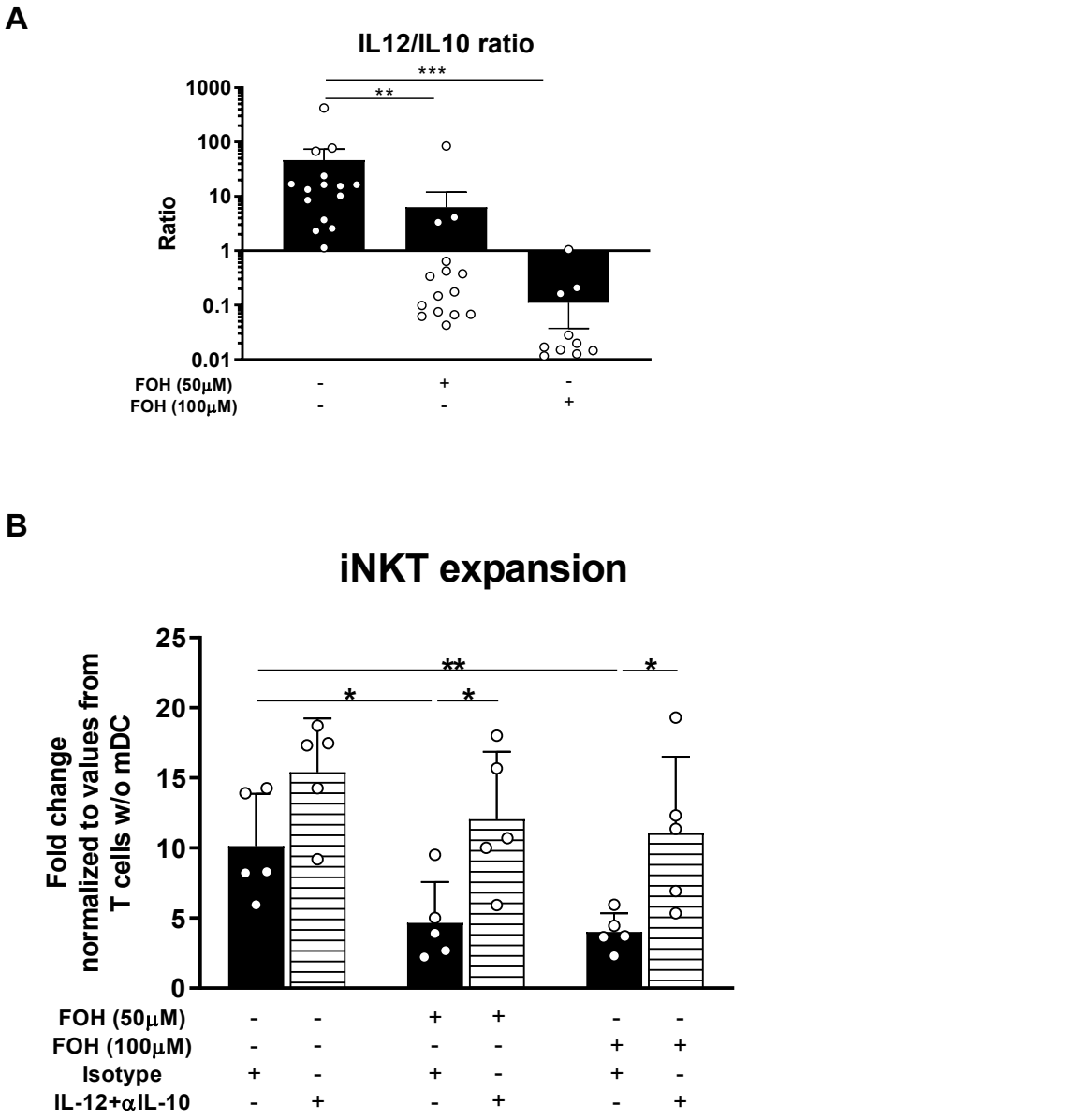

Figure 25. Diminished capacity to induce iNKT expansion by FOH-differentiated mDC is dependent on the altered secretion of IL-12 and IL-10

iDC were differentiated either in the absence or presence of FOH (50 µM and 100 µM), stimulated with LPS for 24 hours, and then supernatants were collected. (A) Data show the ratio between the measured concentrations of IL-12 and IL-10. (B) iNKT expansion was analyzed by flow cytometry after 7 days of co-culture with mock-treated or FOH-instructed mDC in the presence of recombinant IL-12 and anti-IL-10 mAb or the respective isotype control. Bars are means ± SD from 4 independent experiments using cells isolated from different donors and show fold change values acquired between % iNKT cells of total T cells in co-culture with mock- or FOH-treated mDC normalized to values observed in T cells cultured without mDC (*P < 0.05; **P < 0.01, ***P < 0.001).

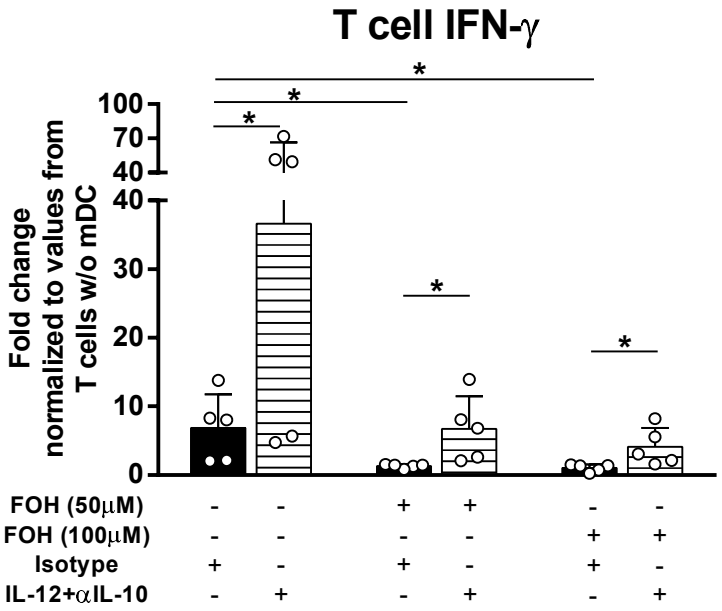

Figure 26. Altered release of IL-12 and IL-10 by FOH-treated mDC impacts their capacity to induce T helper 1 polarization

Autologous T cells were co-cultured with mock-treated or FOH-differentiated mDC in presence or absence of IL-12 and anti-IL-10 mAb, or an isotype control, and IFNγ production by T cells was evaluated after 7 days of co-culture. Bars are means ± SD from 4 independent experiments using cells isolated from different donors and show fold change values between % IFNγ+ T cells of total T cells in co-culture with mock- or FOH-treated mDC and normalized to values observed in T cells cultured without mDC (*P < 0.05).

6.4.2 Farnesol-differentiated DC have a higher expression of inhibitory receptors but lack capacity to induce regulatory T cells expansion

The low expression of costimulatory molecules, together with a higher secretion of IL-10 and reduced capacity of FOH-differentiated mDC to activate iNKT and Th1 cells, might indicate that FOH treatment shift DC functionality towards a tolerogenic phenotype. Therefore, analysis of inhibitory receptors expression, including Ig-like transcripts (ILTs, also called leukocyte Ig-like receptors) ILT2, ILT3, ILT4, and programmed cell death 1 ligand 1 (PD-L1), known to be a hallmark of tolerogenic DC (107, 109), was performed by flow cytometry after differentiation and 24 hours LPS stimulation. The expression of ILT2 and ILT4 was not influenced by FOH (Figure 27). Nevertheless, increased surface exposure of ILT3 after LPS stimulation was evidenced in FOH-differentiated mDC compared to mock-treated cells (mDC with 100 µM FOH, median of 12000 ± 1032, mock-treated mDC, median of 3716 ± 549; $P < 0.001$) Similarly, expression of PD-L1 was enhanced in mDC differentiated in presence of FOH (mDC with 100 µM FOH, median of 4740 ± 151; mock-treated mDC, median of 3136 ± 482; $P < 0.001$). Elevated expression of inhibitory receptors and IL-10 secretion was hypothesized to enhance the capacity to activate $FOXP3^+$ Treg cells (104, 207). Thereby, T cells were co-cultured with mDC differentiated in absence or presence of FOH and Treg cells expansion was evaluated after 7 days. Interestingly, the enhanced expression of inhibitory receptors in FOH-differentiated mDC did not lead to elevated Treg cells expansion compared to mock-treated mDC (mDC with 100 µM FOH, fold change 1.8 ± 1.5; mock-treated mDC, fold change 13.6 ± 10.5) (Figure 28). In line with our previous results, the addition of recombinant IL-12 and IL-10 blocking antibody restored FOH-differentiated DC capacity to activate Treg cells to comparable levels observed in mock-treated mDC co-culture (mDC with 100 µM FOH and IL-12/αIL-10, fold change 11.6 ± 6.5).

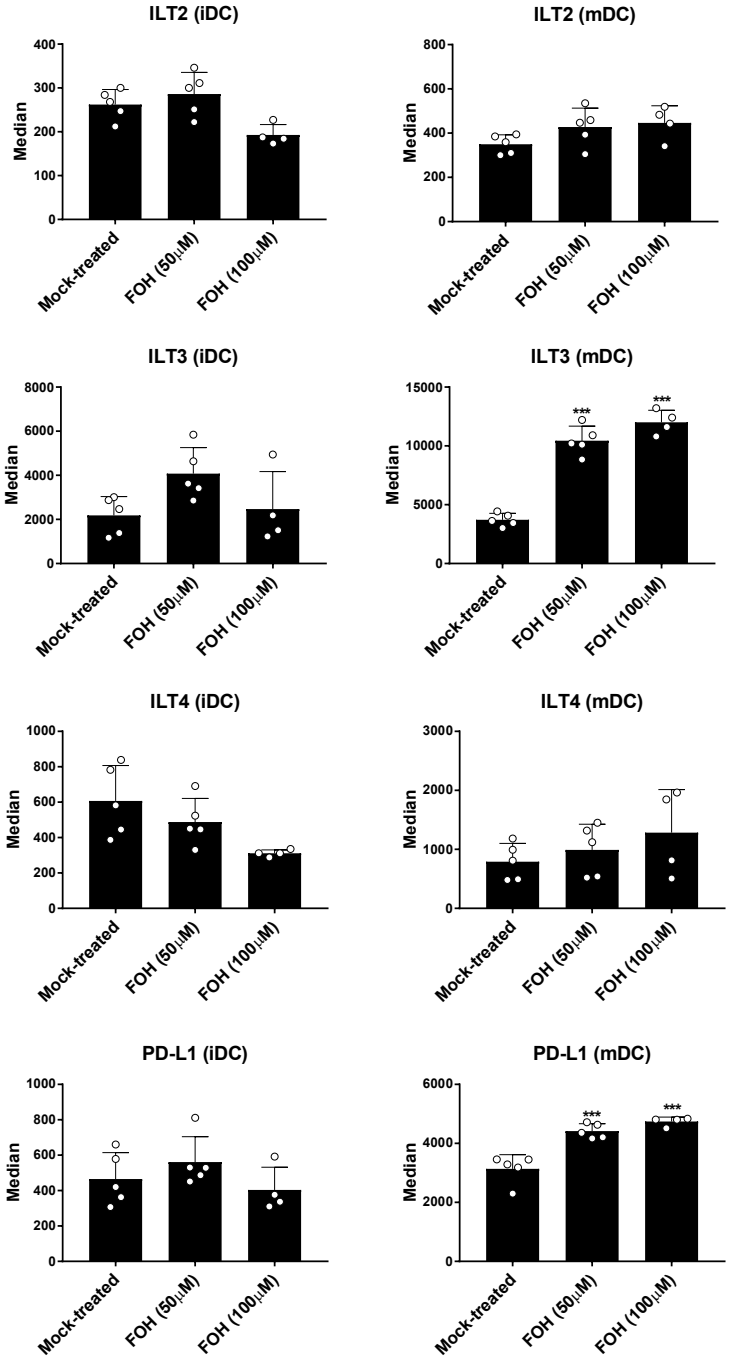

Figure 27. FOH enhances the surface expression of inhibitory receptors on mDC

Monocytes were differentiated into iDC in the presence or absence of FOH followed by a 24 hours LPS stimulation to generate mDC. The expression of ILT2, ILT3, ILT4 and PD-L1 was analyzed by flow cytometry. Quantitative data are means ± SD from at least 4 independent experiments with cells isolated from different donors (***$P < 0.001$ compared to mock-treated).

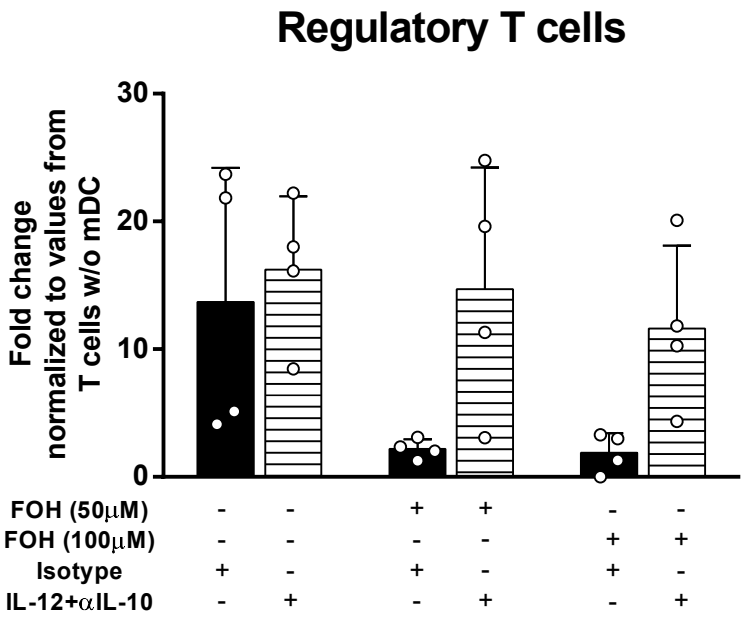

Figure 28. FOH altered DC capacity to induce FOXP3+ regulatory T cells expansion

Autologous T cells were co-cultured with mDC for 7 days in the absence or presence of recombinant IL-12 and anti-IL-10 mAb, or the respective isotype control and $CD4^+CD25^+FOXP3^+$ Treg cells expansion was measured by flow cytometry. Quantitative data are means ± SD from 4 independent experiments with cells isolated from different donors (***$P < 0.001$).

7 Discussion

QS systems coordinate the expression of genes involved in several processes, including virulence in pathogenic microorganisms (15, 208). FOH is the first QS molecule described in a eukaryotic microorganism, and its role in modulating filamentation in *C. albicans* is well understood (27-32). Similarly, FOH has been shown to regulate functionality of innate immune cells, such as neutrophils, monocytes, macrophages and dendritic cells. Leonhardt *et al.* showed that FOH has a profound impact on dendritic cells differentiation from monocytes, while suppressing their capacity to induce T cell proliferation. However, the molecular basis behind these effects is poorly understood (56).

The aim of this study was to analyze the signaling pathways modulated by FOH during dendritic cells differentiation, and its impact on their function.

7.1 Farnesol impairs DC differentiation and maturation partially through nuclear receptors activation

Leonhardt *et al.* found that FOH restrict the differentiation from monocytes to DC, by retaining the expression of the monocyte marker CD14 while impairing costimulatory molecules surface exposure and T cell activation capacity (56). This study confirmed that FOH blocked maturation of DC by modifying the expression of costimulatory and antigen-presenting molecules, as well as the release of cytokines and chemokines in response to *C. albicans* germ tubes and several ligands to Toll-like receptors and dectin-1. These results suggest that FOH-induced changes are not dependent of any specific surface receptor and affect intracellular signaling cascades. Pathway analysis of differentially expressed genes during DC differentiation identified several intracellular processes that were modified in presence of FOH, including the PPAR signaling pathway (56). In agreement, transcriptional analysis revealed that several target genes associated to activation of NR PPARγ, RARα and LXRα were upregulated. Moreover, differentiation of DC in presence of specific ligands for these NR showed a similar phenotype compared to FOH-treated DC. Furthermore, the presence of PPARγ and RARα antagonists blocked upregulation of CD1d by FOH-treatment, and partially reverted FOH effect on CD80 and CD86 surface molecules expression and the release of the chemokine RANTES. Interestingly, RARα and LXRα

showed to have a major role in the release of pro-inflammatory TNF-α induced by FOH. While these results suggest that FOH activates NR, it is not clear how these cells sense FOH in the environment. On the one hand, FOH could be interacting directly with NR as suggested by nuclear localization of a fluorescent analog (20). On the other hand, FOH can be metabolized into farnesyl-PP, an intermediate of the mevalonate/cholesterol biosynthetic pathway, which can be further transformed into NR ligands (209). However, Shchepin *et al.* showed that the mode of action of FOH is specific, since changes in its structure blocked its biological activity (20). Interestingly, FOH is not the only QS molecule known to affect DC differentiation. Treatment of DC with the bacterial QS molecule homoserine lactone diminished the expression of the costimulatory molecules CD80 and CD86 (210). Interestingly, the effect of QS molecules on DC differentiation could have an important impact on septic patients. Boontham *et al.* showed that homoserine lactone effects on DC function correlated with elevated presence of this QS molecule in sera of septic patients (211). Although it is not known the role of FOH during sepsis, Ryan *et al*. showed that monocyte-derived DC from autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) had impaired DC maturation (212). Similar to our results, these DC showed enhanced production of TNF-α and IL-8 and reduced capacity to prime T cells (56, 212).

7.2 Farnesol regulation of CD1 molecules and its functional impact

The most prominent effect observed after dendritic cells differentiation in presence of FOH was the displacement in the expression of CD1 molecules. While CD1a expression was not upregulated, CD1d was enhanced during DC differentiation in the presence of FOH (56). CD1 molecules present lipid antigens to different T cells subsets. In particular, CD1a are specialized in presenting lipid antigens to T cells, while CD1d present antigens to iNKT cells (85). iNKT cells are a subpopulation of T cells with the capacity to modulate immune responses due to its capability to produce high amounts of pro-inflammatory and anti-inflammatory cytokines. Thereby, the shift in the CD1 molecules expression pattern may influence the outcome of infection, since iNKT cells are known to be important during infection against pathogenic microorganisms (213-215). However, there are discrepancies regarding the role of iNKT cells during *C. albicans* infection. Cohen *et al.* showed that iNKT cells activation reduces disease progression in a murine model of systemic candidiasis

(213). Similarly, iNKT cells recognize *C. albicans* α-manosyl residues in a CD1d-dependent manner and increases the survival of mice co-infected with *S. pneumoniae* (215). Nevertheless, different reports showed that iNKT cells have no role or that activation of these cells promotes susceptibility to systemic *C. albicans* infection due to high IL-10 production (216, 217). Thus, more research to elucidate the role of iNKT cells during *C. albicans* infection is needed. While a higher expression of CD1d correlates with an elevated capacity to activate iNKT cells, we failed to observe an enhanced expansion of these cells when co-cultured with FOH-differentiated mDC (87, 156). One possibility could be that FOH-differentiated DC have an impaired capacity to present antigens to T cells. Nakken *et al.* showed that endosomal processing of antigens by proteases is necessary for proper induction of iNKT expansion by DC (178). However, the antigen used in this study (α-GalCer) does not require preprocessing and readily binds to CD1d complex (218). Thereby, a different mechanism should be involved in the reduced capacity of FOH-mDC to activate iNKT cells. In concert with signals triggered by CD1d-mediated lipid presentation, costimulatory molecule interactions between DC and T cells, such as CD40:CD40L and CD80/CD86:CD28, and the release of IL-12 by DC is required for optimal iNKT expansion (219, 220). Indeed, a reduction in CD40 and CD80 was observed in DC differentiated with FOH. However, our results indicate that the shift in the IL-12/IL-10 secretion ratio play an essential role in FOH-differentiated mDC capacity to induce iNKT expansion, since reconstitution of IL-12 and IL-10 to levels observed in absence of FOH significantly improves DC capability to expand iNKT cells.

This work gives insights into the molecular mechanisms by which FOH modulates CD1d expression in DC. Our results showed that FOH regulates CD1d expression via activation of the PPARα/RARα signaling pathway. Szatmari *et al.* demonstrated that activation of PPARγ leads to upregulation of retinaldehyde dehydrogenases (RALDH, ALDH1) that catalyze the production of all-trans retinoic acid (ATRA) (156). ATRA serves as a ligand for RARα which increases CD1d expression in human DC (156). However, proper lipid antigen processing and loading to CD1d molecules requires activation of the lysosomal aspartyl protease cathepsin D CTSD (178). Concurrent with the higher CD1d surface exposure on FOH-differentiated DC, analysis of the transcriptional data identified increased gene expression of NR target genes, including ALDH1A1 and CTSD. Our results confirm previous observations that FOH activates NR, such as PPARγ (209, 221-223). Mechanistically, FOH

could interact directly or indirectly with NR (209, 224). Interestingly, our data suggest that FOH could regulate NR activities through activation of MAPK signaling pathway. Indeed, inhibition of p38 MAPK partially restored CD1d basal levels in FOH-differentiated mDC. NR activities can be modulated independently of the presence of ligands through activation of the AF-1 N-terminal region, which serves as target for post-translational modifications, including phosphorylation (127, 128, 130, 131). Moreover, p38 inhibition showed to diminished PPARγ transcriptional activities in different cell types (225, 226). Finally, we found that FOH-differentiated DC have a higher activation of p38 MAPK compared to mock-treated DC. Altogether, these results indicate that FOH modulates CD1d surface expression via activation of the PPARγ/RARα signaling pathway in a process partially dependent on p38 MAPK.

7.3 Farnesol alters cytokines release through several pathways

The results presented in this study confirm that FOH modulates cytokines and chemokines release by mDC. While production of pro-inflammatory cytokines/chemokines (e.g. TNF-α, IL-8, MCP-1), and the anti-inflammatory cytokine IL-10 was enhanced in FOH-differentiated mDC, T cell activating cytokine and chemokine (e.g. IL-12 and RANTES) was reduced after LPS stimulation. We found that FOH modulates the release of cytokines through a complex activation of NR, MAPK and NF-κB signaling pathways. In particular, TNF-α production in FOH-differentiated mDC was blocked in the presence of inhibitors for JNK MAPK, NF-κB, RARα and LXRα. Accordingly, Szanto *et al.* showed that RARα activation by natural and synthetic ligands promotes the expression of genes such as CYP27A1, an enzyme involved in ligand production for LXRα (227). Activation of LXRα also activates NF-kB, which enhances the secretion of pro-inflammatory TNF-α (191). Interestingly, the elevated release of anti-inflammatory IL-10 observed in FOH-differentiated mDC might be a possible mechanism to suppress the inflammatory milieu observed in our experiments. Indeed, IL-10 production was blocked when p38 MAPK and NF-κB inhibitors were present in the culture and correlated with the secretion of pro-inflammatory cytokines and chemokines observed in FOH-differentiated mDC. Moreover, analyses of p38 MAPK and NF-κB phosphorylation confirmed that FOH-instructed mDC have a higher activation of these pathways compared to mock-treated mDC. However, the diminished secretion of IL-12 and RANTES indicate that FOH-instructed mDC have a reduced T cell activation capacity. Although RANTES

production was partially dependent on PPARγ activation, our results suggest that FOH suppresses IL-12 release through a MAPK, NF-κB and NR independent mechanism. IL-12 gene expression is regulated by MAPK/AP-1, NF-κB and IRF signaling pathways (228). Previous results showed that IL-12p70 release was reduced in IRF-1 deficient DC (228, 229). Moreover, TLR4 activation leads to IRF-3 recruitment to the IL-12p35 promoter, which enhances IL-12p70 synthesis (230). Thus, it is possible that FOH impairs IL-12p70 production in mDC by modulating IRF activation. Interestingly, other microbial QS molecules are known to affect IL-12 release. Homoserine lactone and the *Pseudomona aeruginosa* quinolone signal have been shown to decrease IL-12 production by murine DC (210). Moreover, another molecule produced by *C. albicans*, CA-SIIF, is known to reduce CD1a expression and IL-12 secretion in human DC (231). These results suggest that IL-12 reduction might be a mechanism used by *C. albicans* to overcome immune responses orchestrated by DC.

In summary, our results showed that FOH promotes inflammation, while reduces T cell inducing cytokines and chemokines, in a process dependent on several signaling pathways, including NR, MAPK and NF-κB.

7.4 Farnesol-treated DC have a lower capacity to activate different T cell subsets

DC are considered to be the bridge between the innate and adaptive branch of immunity, due to its potent capacity to present antigens and consequently activating T cell-specific responses (65). The altered expression of costimulatory molecules and cytokines release induced by FOH might indicate an impaired capacity of FOH-differentiated DC to activate T cells. Indeed, FOH-instructed mDC have a lower capability to activate Th1, iNKT and Treg cells. IFN-γ produced by Th1 lymphocytes is essential for stimulation of antifungal activity by neutrophils (97). The role of IFN-γ in anti-Candida immunity is highlighted by the enhanced mortality observed in IFN-γ-deficient mice infected with *C. albicans* and the elevated susceptibility of HIV-infected individuals with low $CD4^+$ T cell count to develop oropharyngeal candidiasis (232, 233). Moreover, IFN-γ contributes to antifungal immunity by inducing nitric oxide production by macrophages, while reducing *Candida*-specific immunoglobulins (234). In agreement with our data, the bacteria QS molecule homoserine

lactone and *P. aeruginosa* quinolone signal has been shown to alter DC capacity to induce activation of T cells (210, 211). However, this is the first report that shows a negative effect of QS molecules in DC capacity to activate FOXP3$^+$ Treg cells. While Th1 cells orchestrate antifungal immunity mainly by activating phagocytes, Treg are involved in controlling inflammation by suppressing the secretion of pro-inflammatory cytokines (235). Indeed, Treg expand during disseminated infection and produce high amounts of IL-4, TGF-β and IL-10, which blocks Th1 immunity (236, 237). Surprisingly, Treg could improve immunity against pathogenic fungi via an alternative mechanism. Treg was shown to promote Th17 differentiation during systemic candidemia and oropharyngeal candidiasis (98, 236). IL-17 play an important role in anti-fungal immunity and exerts its function mainly through recruitment and activation of neutrophils (98, 238). The reduced expression of costimulatory molecules observed in FOH-instructed mDC might influence the impaired capability of these cells to activate T cells. However, our results showed that surface molecules play a minor role in DC capability to induce Treg activation, since the enhanced expression of inhibitory receptors, such as ILT3 and PD-L1 did not correlate with increased FOXP3$^+$ Treg expansion. Reconstitution of IL-12 and IL-10 release to normal levels was sufficient to restore the capacity of FOH-differentiated mDC to activate Th1 and Treg populations. IL-12 is an important cytokine involved in the differentiation and activation of T cells. IL-12 signals through the IL-12 receptor, triggers activation of the Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2), leading to phosphorylation of family members of the STAT transcription factor, resulting in polarization of T cells into INF-γ producing Th1 cells (239). Interestingly, IL-12 signaling increased IFN-γ production in Treg while retaining FOXP3$^+$ expression (240).

Altogether, our results confirm that FOH acts as a compound with immune modulatory properties that blocks the capacity of DC to induce protective adaptive immunity. Since T cells play an important role during *C. albicans* infection, our results support the role of FOH as a virulence factor produce by this fungus to evade DC surveillance.

8 Conclusions

Besides its well-known quorum sensing properties, FOH has been shown to be a modulator of immune function. FOH is able to activate monocytes, neutrophils and macrophages. Interestingly, this molecule altered DC differentiation and maturation while reducing their capacity to activate T cells. Due to the relevance of DC in orchestrating immune responses during infection, identification of the molecular mechanisms by which FOH regulates DC function could lead to possible targets for pharmacological strategies against *C. albicans* infection. Our findings showed for the first time that FOH modulates DC differentiation and maturation through activation of the nuclear receptors PPARγ, RARα and LXRα, MAPK and NF-κB signaling pathways (Figure 29). FOH activates the PPARγ/RARα axis to increase CD1d surface expression on DC in a process partially dependent on p38 MAPK signaling. We found that FOH increased the release of pro-inflammatory cytokines and anti-inflammatory IL-10, and impaired IL-12 release mainly through the RARα, LXRα, MAPK and NF-κB signaling pathways. Despite multiple effects of FOH that in isolation suggest activation of DC, the net effect of FOH exposure is paralysis of DC function due to the reduced ability to prime several T cell subsets. In agreement, the altered secretion of IL-12 and IL-10 induced by FOH treatment showed to play a key role in FOH-differentiated DC capacity to promote iNKT, Th1 and Treg cell activation. Due to the relevance of these cells in the anti-Candida immunity, besides its quorum sensing function, FOH can be viewed as a virulence factor enabling *C. albicans* to overcome the immune surveillance by DC.

Further studies should focus in elucidating the molecular mechanisms underlying the diminished production of IL-12 in FOH-differentiated DC. One possibility might be to evaluate the IRF signaling pathway which is known to modulate IL-12 release in DC (228-230). Furthermore, it would be interesting to characterize the interaction with *Candida albicans* in respect to phagocytosis and killing capacity. The current work shows evidences of the crosstalk between host nuclear receptors and molecules produced by *C. albicans*. Interestingly, fungi present nuclear-receptor like transcription factors (241, 242); thus, it would be interesting to elucidate the role of these transcription factors in the virulence of *C. albicans* and evaluate how molecules produced by host cells might interact with these transcription factors.

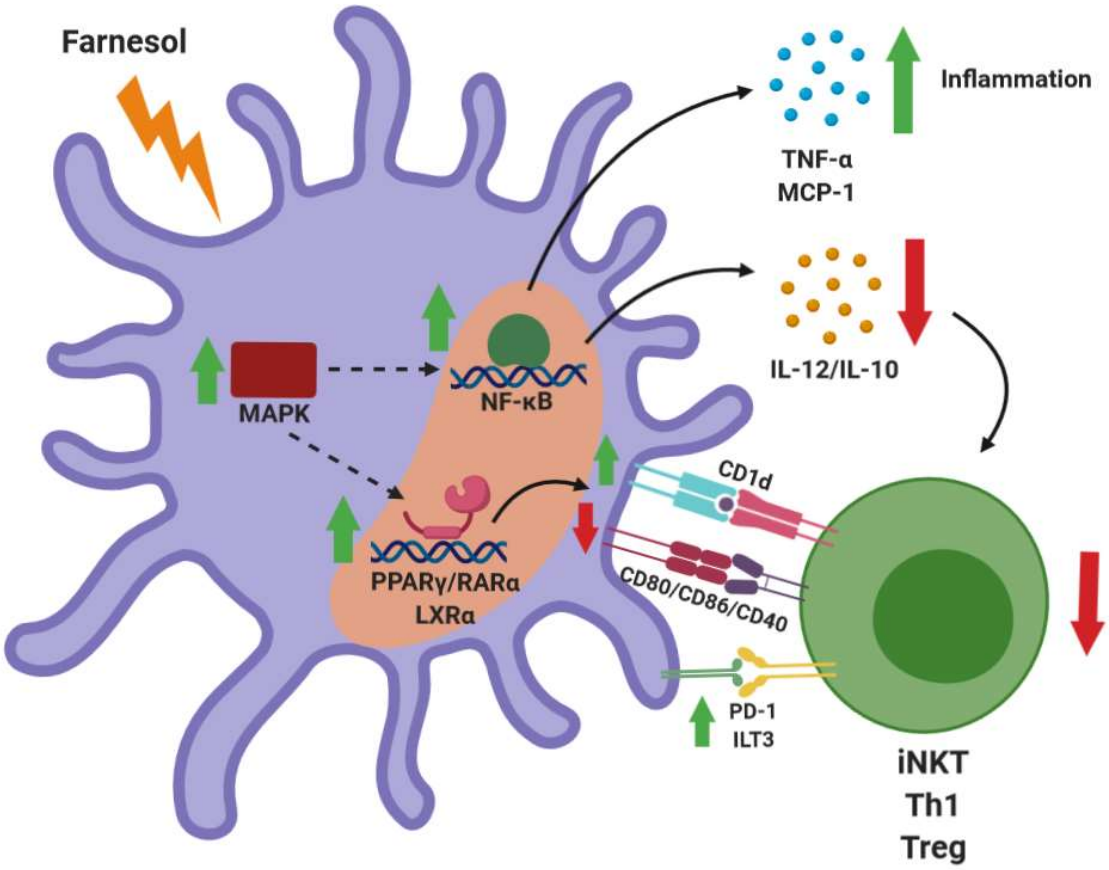

Figure 29. Farnesol modulates DC function through a coordinated activation of several signaling pathways

FOH activates several intracellular signaling pathways resulting in a distinct DC subset, with altered expression of antigen-presenting and costimulatory molecules. FOH modulates the expression of CD1d via p38 MAPK-PPARγ/RARα signaling pathway. Moreover, FOH-differentiated DC showed an elevated secretion of pro-inflammatory and anti-inflammatory IL-10, through MAPK and NF-κB activation. Finally, FOH treatment reduces DC capacity to activate different T cell subsets, such as Th1, iNKT and Treg cells, via reduction of the IL-12/IL-10 secretion ratio.

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10 List of abbreviations

%	Percent
°C	Degree Celsius
Ab	Antibody
AGN	RARα antagonist
AP-1	Activator protein 1
APC	Allophycocyanin
AM	RARα agonist
CD	Cluster of differentiation
CLR	C-type lectin receptors
DC	Dendritic cells
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DPP3	Diacylglycerol pyrophosphate phosphatase
e.g.	exempli gratia
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
et. al.	et alii
FBS	Fetal bovine serum
FCS	Forward scatter
FITC	Fluorescein isothiocyanate
FOH	Farnesol
FOXP3	Forkhead box P3
g	Acceleration due to gravity
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK	LXR antagonist
GW	PPARγ antagonist
HLA-DR	Human leukocyte antigen-D related
iDC	Immature dendritic cells
IFN	Interferon
IL	Interleukin
ILT	leukocyte Ig-like receptors
iNKT	Invariant Natural Killer T cells
JNK	c-Jun N-terminal kinase
LXR	Liver X receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
mDC	Mature dendritic cells
MHC	Major histocompatibility complex
mins	Minutes
MOH	Methanol
MOI	Multiplicity of infection
NFAT	Nuclear factor activated T-cells
NF-κB	Nuclear factor kappa B
NR	Nuclear receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PD-L	Programmed death-ligand
PE	Phycoerythrin
PerCP	Peridinin chlorophyll

PMA	Phorbol 12-merystate 13-acetate
PPAR	Peroxisome proliferator activated receptor
PRR	Pathogen recognition receptor
QS	Quorum sensing
RANTES	Regulated upon activation, normally T cell expressed and secreted
RAR	Retinoic acid receptor
RXR	Retinoic X receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RSG	Rosiglitazone: PPARγ agonist
STAT	Signal transducer and activators of transcription
SD	Standard deviation
TGF-β	transforming growth factor beta
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
Th17	Type 17 helper T cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cells
U	Units
V450	Violet 450 nm
Vs	Versus
YPD	Yeast peptone dextrose
α	Alpha
β	Beta
γ	Gamma
µM	Micromolar

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12 Appendix

12.1 Curriculum Vitae

Wolfgang Vivas

Date of birth:	January 18, 1990
Place of birth:	Caracas, Venezuela
Nationality:	Venezuelan

Education

Dr. rer. nat.	**Friedrich Schiller University Jena**, Jena - Germany (2015-2020)
Licenciado en Biologia (Diploma in Biology)	**Universidad Central de Venezuela**, Caracas - Venezuela (2007 - 2013)

Research Experience

2015 –2019 **Doctoral Researcher**

Project: „Modulation of dendritic cell function by the fungal quorum sensing molecule farnesol”

Supervisor: Prof. Oliver Kurzai.

- Phenotypic and functional characterization of dendritic cells differentiated in presence of farnesol
- Determined the contribution of nuclear receptors in the expression of surface molecules and cytokines release of dendritic cells treated with farnesol
- Examined the role of MAPK and NF-κB signaling pathways in the altered functionality of dendritic cells stimulated with farnesol
- Evaluation of T cells activation by farnesol-instructed dendritic cells

2014 – 2015 **PhD student**

Project: “Role of protease-activated receptors in hemostasis”

Supervisor: Dr. Mercedes Lopez

- Isolation and culture of natural killer T cells from healthy donors
- Determination of cytokines from natural killer T cells stimulated with protease-activated receptors agonists

2010 – 2013:	**Undergraduate thesis student** Project: "Role of regulatory T cells in the immune response against BCG in HIV-infected children under HAART treatment" Supervisor: Prof. Miguel Alfonzo • Isolation and culture of T cells and regulatory T cells from HIV-infected children under HAART treatment • Determined the impact of HAART treatment in the phenotypic and functional properties of $CD4^+$ and $CD8^+$ T cells against BCG from HIV-infected children

Research Supports

2015 – 2018	PhD fellowship from the Jena School of Microbial Communication (JSMC)
2014 – 2015	PhD scholarship from the Venezuelan Institute for Scientific Research (IVIC)

Committee Leadership

2016 – 2018	JSMC doctoral researchers' representative
2011 – 2013	Biology student's representative at the faculty board

Awards

2014	Venezuelan representation and winning team of the Novartis International Biotechnology Leadership Camp 2014
2013	Thesis dissertation with honors. Universidad Central de Venezuela

Publications

- Wolfgang Vivas, Ines Leonhardt, Kerstin Hünniger, Antje Häder, Alessandra Marolda, Oliver Kurzai. (2019). *Multiple Signaling Pathways Involved in Human Dendritic Cell Maturation Are Affected by the Fungal Quorum-Sensing Molecule Farnesol.* **J Immunol,** 2019, 2013 (11) 2959-69.
- Alessandra Marolda, Kerstin Hünniger, Sarah Böttcher, Wolfgang Vivas, Jürgen Löffler, Marc Thilo Figge, Oliver Kurzai. *Candida species-dependent release of IL-12 by dendritic cells induces different levels of NK cell stimulation.* **J Infect Dis**. 2020, 2060-71.

Selected Presentations

Oral

- **52th Wissenschaftliche Tagung der Deutschsprachigen Mykologischen Gesellschaft e. V**. Innsbruck, Austria. 06 - 08.09.2018. *Candida albicans* quorum sensing molecule farnesol activates nuclear receptors and modulates dendritic cells functionality. Wolfgang Vivas, Ines Leonhardt, Oliver Kurzai
- **7th International Conference on Microbial Communication for Young Scientists**. Jena, Germany, 19 - 22.03.2018. *Candida albicans* quorum sensing molecule farnesol modulates human dendritic cells function through the PPARg pathway. Wolfgang Vivas, Ines Leonhardt, Oliver Kurzai

Poster

- **20th Congress of the International Society for Human and Animal Mycology**. Amsterdam, Netherlands. 30.06.2018 - 04.07.2018. *Candida albicans* quorum sensing molecule farnesol modulates human dendritic cells function by nuclear receptors activation. Wolfgang Vivas, Ines Leonhardt, Oliver Kurzai
- **47th Jahrestagung der Deutschen Gesselchaft für Immunologie.** Erlangen, Germany. 12 – 15.09.2017. The fungal quorum sensing molecule Farnesol modulates CD1 molecules expression in dendritic cells. Wolfgang Vivas, Ines Leonhardt, Oliver Kurzai
- **Annual Conference of the Association for General and Applied Microbiology (VAAM).** Jena, Germany. 13 – 16.03.2016. The fungal quorum sensing molecule farnesol impairs dendritic cells maturation. Wolfgang Vivas, Ines Leonhardt, Oliver Kurzai

Spoken Languages

Spanish (native proficiency)
English (C2 level/proficient user)
German (A2 level/basic user)

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12.3 Declaration of originality

I herewith declare that I am familiar with the relevant course of examination for doctoral Candidates of the Faculty of Biological Sciences of the Friedrich Schiller University, Jena. I declare that I, personally, composed and wrote this dissertation and that I have acknowledged any and all additional assistance, personal communications and sources according to the rules of academic work. I declare that assistance provided by specific individuals during the study and writing of this dissertation has been indicated in full. I declare that I did not enlist any assistance of a doctoral consultant and that no third parties have received, either directly or indirectly, monetary benefits from me for work connected to this submitted dissertation. I declare that this dissertation has not been submitted as an examination paper for a state, or other, scientific examination. I also declare that I did not submit the same, a substantially similar or a different dissertation to another education body.

I am aware that a false declaration will have legal consequences.

……..

Wolfgang Vivas